(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 191 099 A1

(12) EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:
27.03.2002 Bulletin 2002/13

(21) Application number: **00942387.2**

(22) Date of filing: **29.06.2000**

(51) Int Cl.[7]: **C12N 15/12**, C12N 5/10, C12P 21/02, C07K 14/47, C07K 16/18, A61K 45/00, A61K 38/17, A61K 39/395, A61K 49/16, A61P 19/02, A61P 19/08, A61K 31/7088 // C12P21:02, C12R1:19

(86) International application number:
PCT/JP00/04278

(87) International publication number:
WO 01/02564 (11.01.2001 Gazette 2001/02)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **30.06.1999 JP 18671899**

(71) Applicant: **Takeda Chemical Industries, Ltd.**
Osaka-shi, Osaka 541-0045 (JP)

(72) Inventors:
- **ITOH, Yasuaki**
  Tsuchiura-shi, Ibaraki 300-0832 (JP)
- **NISHI, Kazunori**
  Tsukuba-shi, Ibaraki 305-0044 (JP)
- **OGI, Kazuhiro**
  Tsukuba-shi, Ibaraki 305-0045 (JP)
- **OHKUBO, Shoichi**
  Usiku-shi, Ibaraki 300-1234 (JP)
- **MOGI, Shinichi**
  Kitasoma-gun, Ibaraki 302-0121 (JP)
- **NOGUCHI, Yuko**
  Ibaraki 305-0051 (JP)
- **YOSHIMURA, Koji**
  Tsukuba-shi, Ibaraki 305-0821 (JP)
- **TANAKA, Hideyuki**
  Tsukuba-shi, Ibaraki 305-0821 (JP)

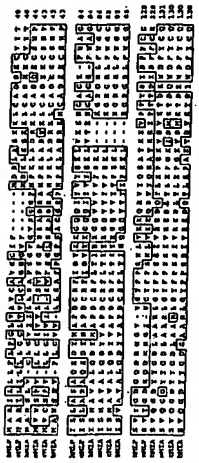
(74) Representative: **Keller, Günter, Dr. et al**
Lederer & Keller
Patentanwälte
Prinzregentenstrasse 16
80538 München (DE)

(54) NOVEL POLYPEPTIDE AND DNA THEREOF

(57) The present invention relates to novel polypeptides and DNAs encoding the same, pharmaceutical compositions comprising the polypeptides or the DNAs, a screening method/screening kit for compounds or salts thereof that promote or inhibit the activity of the polypeptides, medicaments comprising the compounds or salts thereof, etc.

The polypeptides of the present invention and DNAs encoding the same can be used for the diagnosis, treatment, prevention, etc. of, e.g., bone and joint diseases or pathological angiogenesis. Also, the polypeptides of the present invention are useful as reagents for screening the compounds or salts thereof that promote or inhibit the activity of the polypeptides.

Fig.1



EP 1 191 099 A1

Description

FIELD OF THE INVENTION

[0001] The present invention relates to a novel secretory cell function regulating protein and its DNA.

BACKGROUND ART

[0002] Regardless of prokaryotic or eukaryotic, cells secret various proteins through mechanisms inherent to them. In particular, a multicellular organism (living body) exchanges intercellularly a variety of signals to maintain its differentiation, proliferation and homeostasis, and various humoral factors that play pivotal roles for the signal transduction are mostly secretory proteins or mature proteins. These factors are classified into hormones, neurotransmitters, cytokines, growth factors, etc., based on the structural and functional properties. Through the advanced recombinant DNA technology and cell culture technology in recent years, it has been steadily proceeded to clarify genes encoding these proteins and their protein structures. On the other hand, discovery of such factors have made a breakthrough in analysis of receptors of these factors expressed on the cell surface and further have led to clarification of mechanism on intracellular signal transduction, which will characterize the physiological function of the cells. In many diseases in human or under pathological conditions of model animal with various diseases, it is often found that abnormal expression of some humoral factor that should normally maintain homeostasis causes these diseases, which, as a result, will lead to exacerbation. In addition, there is a phenomenon applicable to diagnosis of various diseases, such as a so-called tumor marker, in which the accentuated expression specifically observed in cancer, and its expression-controlling mechanism is also an important target in pharmaceutical discovery research.

[0003] MIA (melanoma inhibitor activity) reported by Blesch et al. in 1994 is one of the secretory proteins falling within such a category. At first, MIA was isolated from the culture supernatant of melanoma cells using an anti-proliferating activity against human melanoma cells as is seen from its name, and its gene was also acquired (Cancer Research, 54, 5695-5701, 1994). Subsequently in 1996, a homologous gene to this protein was again identified by Sandell et al. as CD-RAP (bovine cartilage-derived retinoic acid-sensitive protein), suggesting that CD-RAP will function to form and maintain the formation and maintenance of joints from a physiological aspect (The Journal of Biological Chemistry, 271, 3311-3316, 1996). Though the MIA/CD-RAP gene has a homology as high as 85% or more between species of human mouse, rat and bovine, any known homologous protein has not been found so far. From genetic analysis in bovine and rat, it was also considered that there was no other gene similar to the MIA/CD-RAP gene (The Journal of Biological Chemistry, 271, 3311-3316, 1996).

[0004] On the other hand, structural analysis of the full-length DNA one organism possesses, i.e., genome, has already been decoded in bacteria, and human genome analysis will also be completed in a few years. The predicted number of genes is said to reach 100,000 in human. Indeed, many genes encoding the secretory proteins or secretory peptides have been isolated so far, but on the whole, it cannot be said that even these many genes cover all of the entire genome. In understanding the phenomena of life on an individual level, intercellular signal transduction that would occur there must be all explainable. It is highly likely that some unknown humoral functional molecules other than such known genes may play physiologically critical roles, and it has been strongly desired to find such substances.

[0005] The present invention aims at providing a novel cell function regulating secretory protein (hereinafter sometimes referred to as MLP protein or MLP), its partial peptide or salts thereof, a DNA encoding the protein, a recombinant vector, a transformant, a method for manufacturing the protein, a pharmaceutical composition comprising the protein or the DNA, an antibody to the protein, a method and kit for screening a receptor agonist/antagonist, a receptor agonist/antagonist obtainable by the screening, as well as a pharmaceutical comprising the receptor agonist/antagonist, and the like.

[0006] Isolation of a novel cell function regulating secretory protein can not only lead to a new finding on the mechanism of differentiation, proliferation, malignant alteration, etc., but also can make a further progress to clarify the phenomena of life, including ontogenesis, maintenance of homeostasis, etc. and exhibit an inhibitory activity against or a promoting activity for the protein, resulting in development of a novel pharmaceutical useful for the prevention, diagnosis and treatment of various diseases.

DISCLOSURE OF THE INVENTION

[0007] The present inventors have made extensive studies and as a result, succeeded in cloning cDNAs each having a novel base sequence, from human fetal brain- and mouse fetal brain-derived cDNA libraries. The present inventors have found that proteins encoded by the thus obtained cDNAs are precursor proteins of MIA/CD-RAP-like protein MLP having a useful cell function regulating secretory activity and MLP formed after cleaving signal sequence out of the MLP precursor is a secretory protein. Based on these findings, the present invention have made further investigations

and come to accomplish the present invention.

[0008] That is, the present invention provides the following features.

(1) A polypeptide containing an amino acid sequence, which is the same or substantially the same as the amino acid sequence represented by SEQ ID NO:24, its amide or ester, or a salt thereof.

(2) The polypeptide, its amide or ester, or a salt thereof, according to (1), which comprises an amino acid sequence that is the same or substantially the same as the amino acid sequence represented by SEQ ID NO:6.

(3) The polypeptide or its amide or ester, or a salt thereof, according to (1), wherein substantially the same amino acid sequence represented by SEQ ID NO:24 is the amino acid sequence represented by SEQ ID NO:26.

(4) The polypeptide or its amide or ester, or a salt thereof, according to (2), wherein substantially the same amino acid sequence represented by SEQ ID NO:6 is the amino acid sequence represented by SEQ ID NO:12.

(5) The polypeptide or its amide or ester, or a salt thereof, according to (1), wherein substantially the same amino acid sequence represented by SEQ ID NO:24 is the amino acid sequence represented by SEQ ID NO:49.

(6) The polypeptide or its amide or ester, or a salt thereof, according to (2), wherein substantially the same amino acid sequence represented by SEQ ID NO:6 is the amino acid sequence represented by SEQ ID NO:47.

(7) A DNA containing a DNA bearing a base sequence encoding the polypeptide according to (1).

(8) The DNA according to (6), wherein the base sequence encoding the polypeptide according to (1) is the base sequence represented by SEQ ID NO:23.

(9) The DNA according to (6), wherein the base sequence encoding the polypeptide according to (1) is the base sequence represented by SEQ ID NO:4.

(10) The DNA according to (6), wherein the base sequence encoding the polypeptide according to (1) is the base sequence represented by SEQ ID NO:25.

(11) The DNA according to (6), wherein the base sequence encoding the polypeptide according to (1) is the base sequence represented by SEQ ID NO:10.

(12) The DNA according to (6), wherein the base sequence encoding the polypeptide according to (1) is the base sequence represented by SEQ ID NO:48.

(13) The DNA according to (6), wherein the base sequence encoding the polypeptide according to (1) is the base sequence represented by SEQ ID NO:46.

(14) A recombinant vector comprising the DNA according to (6).

(15) A transformant transformed with the recombinant vector according to claim 14.

(16) A method for manufacturing the polypeptide or its amide or ester, or a salt thereof, according to (1), which comprises culturing said transformant according to (15) and producing the polypeptide according to (1).

(17) An antibody to the polypeptide or its amide or ester, or a salt thereof, according to (1).

(18) A method of screening a compound or its salt that promotes or inhibits the activity of the polypeptide or its salt according to (1), which comprises using the polypeptide, its amide or ester, or a salt thereof, according to (1).

(19) A kit for screening a compound or its salt that promotes or inhibits the activity of the polypeptide, its amide or ester, or a salt thereof, according to (1), comprising the polypeptide or its salt according to (1).

(20) A compound or its salt that promotes or inhibits the activity of the polypeptide, its amide or ester, or a salt thereof, according to (1), which is obtainable using the screening method according to (18) or using the screening kit according to (19).

(21) A pharmaceutical comprising a compound or its salt that promotes or inhibits the activity of the polypeptide, its amide or ester, or a salt thereof, according to (1), which is obtainable using the screening method according to (18) or using the screening kit according to (19).

(22) A pharmaceutical comprising the polypeptide, its amide or ester, or a salt thereof, according to (1).

(23) An agent for the prevention/treatment of bone and joint diseases or pathologic angiogenesis, comprising the polypeptide, its amide or ester, or a salt thereof, according to (1).

(24) A diagnostic agent comprising the antibody according to (17).

The present invention further relates to the following features.

(25) The polypeptide, its amide or ester, or a salt thereof, according to (1), wherein substantially the same amino acid sequence as that represented by SEQ ID NO:24 is an amino acid sequence having homology of at least about 50% (preferably at least about 60%, more preferably at least about 70%, much more preferably at least about 80%, further much more preferably at least about 90%, and most preferably about 95%), to the amino acid sequence represented by SEQ ID NO:24.

(26) The polypeptide, its amide or ester, or a salt thereof, according to (1), wherein substantially the same amino acid sequence as the amino acid sequence shown by SEQ ID NO:24 is (i) an amino acid sequence represented by SEQ ID NO:24, of which 1 or 2 more (preferably approximately 1 to 30) amino acids are deleted; (ii) an amino acid sequence represented by SEQ ID NO:24, to which 1 or 2 more (preferably approximately 1 to 40, more preferably approximately 1 to 30) amino acids are added; (iii) an amino acid sequence represented by SEQ ID

NO:24, in which 1 or 2 more (preferably approximately 1 to 30) amino acids are substituted by other amino acids; and (iv) a combination of the above amino acid sequences.

[0009] Furthermore, the DNA, polypeptide or its amide or ester or a salt thereof, etc. of the present invention can be utilized for basic studies, including molecular weight markers, tissue markers, chromosomal mapping, identification of hereditary diseases, design or primers or probes, etc.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010]

FIG. 1 shows the amino acid sequences of human MLP precursor (hMLP), mouse MLP precursor (mMLP), human MIA precursor (hMIA), mouse MIA precursor (mMIA), rat MIA precursor (rMIA) and bovine MIA precursor (bMIA).
FIG. 2 shows the results of Western blotting analysis performed in EXAMPLE 6, in which anti-FLAG antibody was employed as a primary antibody.

[0011] In the figure, Lanes 1, 2, 3, 4, 5 and 6 designate lanes obtained by electrophoresis of the culture supernatants of COS-7 cells introduced with mouse MLP (no FLAG tag), mouse MLP-FLAG, mouse MIA (no FLAG tag), mouse MIA-FLAG, human MLP (no FLAG tag) and mouse MLP-FLAG, respectively.

[0012] FIG. 3 shows the results of Western blotting analysis performed in EXAMPLE 6, in which anti-MLP antibody was employed as a primary antibody.

[0013] In the figure, Lanes 1, 2, 3, 4, 5 and 6 designate lanes obtained by electrophoresis of the culture supernatants of COS-7 cells introduced with mouse MLP (no FLAG tag), mouse MLP-FLAG, mouse MIA (no FLAG tag), mouse MIA-FLAG, human MLP (no FLAG tag) and mouse MLP-FLAG, respectively.

[0014] FIG. 4 shows the results of immunostaining performed in EXAMPLE 6, in which the left panel indicates the result of control experiment using pre-immune rabbit sera and the right panel indicates the results obtained using anti-MLP antisera.

BEST MODE OF EMBODIMENT OF THE INVENTION

[0015] The polypeptide of the present invention containing the amino acid sequence represented by SEQ ID NO:24 (hereinafter sometimes referred to as human type polypeptide), the polypeptide containing the amino acid sequence represented by SEQ ID NO:26 (hereinafter sometimes referred to as mouse type polypeptide), the polypeptide containing the amino acid sequence represented by SEQ ID NO:49 (hereinafter sometimes referred to as rat type polypeptide) and the polypeptide containing an amino acid sequence, which is substantially the same as the human type polypeptide (hereinafter the human type polypeptide and the polypeptide containing an amino acid sequence, which is substantially the same as the human type polypeptide are sometimes collectively referred to as the polypeptide of the present invention) may be any polypeptide derived from any cells of human and other warm-blooded animals (e.g., guinea pig, rat, mouse, chicken, rabbit, swine, sheep, bovine, monkey, etc.) (e.g., liver cells, splenocytes, nerve cells, glial cells, β cells of pancreas, bone marrow cells, mesangial cells, Langerhans' cells, epidermic cells, epithelial cells, endothelial cells, fibroblasts, fibrocytes, myocytes, fat cells, immune cells (e.g., macrophage, T cells, B cells, natural killer cells, mast cells, neutrophils, basophils, eosinophils, monocytes), megakaryocytes, synovial cells, chondrocytes, bone cells, osteoblasts, osteoclasts, mammary gland cells, or interstitial cells; the corresponding precursor cells, stem cells, cancer cells, etc.); or any tissues where such cells are present, such as brain or any of brain regions (e.g., olfactory bulb, amygdaloid nucleus, basal ganglia, hippocampus, thalamus, hypothalamus, cerebral cortex, medulla oblongata, cerebellum), spinal cord, hypophysis, stomach, pancreas, kidney, liver, gonad, thyroid, gallbladder, bone marrow, adrenal gland, skin, muscle, lung, gastrointestinal tract (e.g., large intestine and small intestine), blood vessel, heart, thymus, spleen, submandibular gland, peripheral blood, prostate, testis, ovary, placenta, uterus, bone and joint, skeletal muscle, etc.; the polypeptides may also be recombinant polypeptides or synthetic polypeptides.

[0016] When the polypeptide of the present invention carries a signal peptide, the polypeptide can be extracellularly secreted efficiently.

[0017] The amino acid sequence which is substantially the same as the amino acid sequence represented by SEQ ID NO:24 includes an amino acid sequence having homology of at least about 50%, preferably at least about 60%, more preferably at least about 70%, much more preferably at least about 80%, further much more preferably at least about 90%, and most preferably about 95%, to the amino acid sequence represented by SEQ ID NO:24, and specific examples of such amino acid sequences are the amino acid sequence represented by SEQ ID NO:26, the amino acid sequence represented by SEQ ID NO:49, and the like.

[0018] The polypeptide containing the amino acid sequence represented by SEQ ID NO:24 is sometimes referred

to as human MLP or human MLP protein; the amino acid sequence represented by SEQ ID NO:26 is sometimes referred to as mouse MLP or mouse MLP protein; the amino acid sequence represented by SEQ ID NO:49 is sometimes referred to as rat MLP or rat MLP protein; and these polypeptides are sometimes collectively referred to as MLP.

[0019] Specific examples of the polypeptide containing the amino acid sequence shown by SEQ ID NO:24 or an amino acid sequence which is substantially the same as the amino acid sequence shown by SEQ ID NO:24 include the polypeptide containing the amino acid sequence shown by SEQ ID NO:6 or an amino acid sequence which is substantially the same as the amino acid sequence shown by SEQ ID NO:6, and the like.

[0020] The amino acid sequence which is substantially the same as the amino acid sequence represented by SEQ ID NO:6 includes an amino acid sequence having homology of at least about 50%, preferably at least about 60%, more preferably at least about 70%, much more preferably at least about 80%, further much more preferably at least about 90%, and most preferably about 95%, to the amino acid sequence represented by SEQ ID NO:6, and specific examples of such amino acid sequences are the amino acid sequence represented by SEQ ID NO:12, the amino acid sequence represented by SEQ ID NO:47, and the like.

[0021] The polypeptide containing the amino acid sequence represented by SEQ ID NO:6 is sometimes referred to as human MLP precursor or human MLP precursor protein; the amino acid sequence represented by SEQ ID NO:12 is sometimes referred to as mouse MLP precursor or mouse MLP precursor protein; the amino acid sequence represented by SEQ ID NO:47 is sometimes referred to as rat MLP precursor or rat MLP precursor protein; and they are sometimes collectively referred to as MLP precursors.

[0022] Preferred examples of the polypeptide of the present invention containing an amino acid sequence, which is substantially the same as the amino acid sequence shown by SEQ ID NO:24, include a polypeptide containing an amino acid sequence, which is substantially the same as the amino acid sequence shown by SEQ ID NO:24 and has a property substantially equivalent to that of the polypeptide having the amino acid sequence shown by SEQ ID NO: 24, and the like.

[0023] Preferred examples of the polypeptide containing an amino acid sequence which is substantially the same amino acid sequence represented by SEQ ID NO:6, include a polypeptide containing an amino acid sequence, which is substantially the same as the amino acid sequence shown by SEQ ID NO:6 and has a property substantially equivalent to that of the polypeptide having the amino acid sequence shown by SEQ ID NO:6, and the like.

[0024] The substantially equivalent property includes, for example, an activity that is secreted and acts as a humoral factor, and the like. The term substantially equivalent is used to mean that these activities are equivalent qualitatively. Therefore, it is preferred that activities such as a secretory activity, solubility, etc. are equivalent (e.g., about 0.1 to about 100 times, preferably about 0.5 to about 10 times, more preferably about 0.5 to about 2 times), but it is allowable that differences in quantitative factor such as strength of these activities, molecular weight of the polypeptide may be present.

[0025] More specifically, the polypeptide containing an amino acid sequence, which is substantially the same amino acid sequence represented by SEQ ID NO:24 or SEQ ID NO:6, includes a so-called mutein such.as a polypeptide containing (i) an amino acid sequence represented by SEQ ID NO:24 or SEQ ID NO:6, of which 1 or 2 more (preferably approximately 1 to 30, more preferably approximately 1 to 10 and most preferably several (1 to 5) amino acids have been deleted; (ii) an amino acid sequence represented by SEQ ID NO:24 or SEQ ID NO:6, to which 1 or 2 more (preferably approximately 1 to 40, more preferably approximately 1 to 30, much more preferably approximately 1 to 10 and most preferably several (1 to 5) amino acids have been added; (iii) an amino acid sequence represented by SEQ ID NO:24 or SEQ ID NO:6, into which 1 or 2 more (preferably approximately 1 to 30, more preferably approximately 1 to 10 and most preferably several (1 to 5) amino acids have been inserted, (iv) an amino acid sequence represented by SEQ ID NO:24 or SEQ ID NO:6, in which 1 or 2 more (preferably approximately 1 to 30, more preferably approximately 1 to 10 and most preferably several (1 to 5) amino acids have been substituted by other amino acids; and (v) a combination of the above amino acid sequences, and the like.

[0026] When an amino acid sequence(s) have been inserted, deleted or substituted as described above, the positions of such insertion, deletion or substitution are not particularly limited, but examples of the positions are the position other than that for the amino acid sequence common to the amino acid sequences shown by SEQ ID NO:24, SEQ ID NO:26 and SEQ ID NO:49, the position other than that for the amino acid sequence common to the amino acid sequences shown by SEQ ID NO:6, SEQ ID NO:12 and SEQ ID NO:47, etc.

[0027] Throughout the present specification, the polypeptides are represented in accordance with the conventional way of describing polypeptides, that is, the N-terminus (amino terminus) at the left hand and the C-terminus (carboxyl terminus) at the right hand. In the polypeptides of the present invention including the polypeptide containing the amino acid sequence shown by SEQ ID NO:24, the C-terminus is usually in the form of a carboxyl group (-COOH) or a carboxylate ($-COO^-$) but may be in the form of an amide ($-CONH_2$) or an ester (-COOR).

[0028] Herein, examples of the ester group shown by R include a C_{1-6} alkyl group such as methyl, ethyl, n-propyl, isopropyl, n-butyl, etc.; a C_{3-8} cycloalkyl group such as cyclopentyl, cyclohexyl, etc.; a C_{6-12} aryl group such as phenyl, α-naphthyl, etc.; a C_{7-14} aralkyl such as a phenyl-C_{1-2} alkyl group, e.g., benzyl, phenethyl, etc.; an α-naphthyl-C_{1-2}

alkyl group such as α-naphthylmethyl, etc.; and the like. In addition, pivaloyloxymethyl or the like which is used widely as an ester for oral administration may also be used.

[0029] Where the polypeptide of the present invention contains a carboxyl group (or a carboxylate) at a position other than the C-terminus, it may be amidated or esterified and such an amide or ester is also included within the polypeptide of the present invention. The ester group may be the same group as that described with respect to the above C-terminal.

[0030] Furthermore, examples of the polypeptide of the present invention include variants of the above polypeptides, wherein the amino group at the N-terminus (e.g., methionine residue) of the polypeptide is protected with a protecting group (e.g., a C_{1-6} acyl group such as a C_{1-6} alkanoyl group, e.g., formyl group, acetyl group, etc.); those wherein the N-terminal region is cleaved in vivo and the glutamyl group thus formed is pyroglutaminated; those wherein a substituent (e.g., -OH, -SH, amino group, imidazole group, indole group, guanidino group, etc.) on the side chain of an amino acid in the molecule is protected with a suitable protecting group (e.g., a C_{1-6} acyl group such as a C_{1-6} alkanoyl group, e. g., formyl group, acetyl group, etc.), or conjugated proteins such as glycoproteins having sugar chains.

[0031] The polypeptide of the present invention or salts thereof may be used in the form of salts with physiologically acceptable acids (e.g., inorganic acids or organic acids) or bases (e.g., alkali metal salts), preferably in the form of physiologically acceptable acid addition salts. Examples of such salts are salts with inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid), salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

[0032] The polypeptide of the present invention or salts thereof may be manufactured by a publicly known method used to purify a polypeptide (protein) from human or other warm-blooded animal cells or tissues described above, or may also be manufactured by culturing a transformant containing DNA encoding the polypeptide later described. Furthermore, the polypeptide of the present invention or salts thereof may also be manufactured by a modification of the peptide synthesis method, which will be described hereinafter.

[0033] Where the polypeptide or salts thereof are manufactured from human or mammalian tissues or cells, human or mammalian tissues or cells are homogenized, then extracted with an acid or the like, and the extract is isolated and purified by a combination of chromatography techniques such as reverse phase chromatography, ion exchange chromatography, and the like.

[0034] To synthesize the polypeptide of the present invention, its salts or amides, commercially available resins that are used for polypeptide (protein) synthesis may be used. Examples of such resins include chloromethyl resin, hydroxymethyl resin, benzhydrylamine resin, aminomethyl resin, 4-benzyloxybenzyl alcohol resin, 4-methylbenzhydrylamine resin, PAM resin, 4-hydroxymethylmethylphenyl acetamidomethyl resin, polyacrylamide resin, 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxy resin, 4-(2',4'-dimethoxyphenyl-Fmoc-aminoethyl)phenoxy resin, etc. Using these resins, amino acids in which α-amino groups and functional groups on the side chains are appropriately protected are condensed on the resin in the order of the sequence of the objective polypeptide according to various condensation methods publicly known in the art. At the end of the reaction, the polypeptide is excised from the resin and at the same time, the protecting groups are removed. Then, intramolecular disulfide bond-forming reaction is performed in a highly diluted solution to obtain the objective polypeptide or amides thereof.

[0035] For condensation of the protected amino acids described above, a variety of activation reagents for polypeptide synthesis may be used, but carbodiimides are particularly preferably employed. Examples of such carbodiimides include DCC, N,N'-diisopropylcarbodiimide, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide, etc. For activation by these reagents, the protected amino acids in combination with a racemization inhibitor (e.g., HOBt, HOOBt) are added directly to the resin, or the protected amino acids are previously activated in the form of symmetric acid anhydrides, HOBt esters or HOOBt esters, followed by adding the thus activated protected amino acids to the resin.

[0036] Solvents suitable for use to activate the protected amino acids or condense with the resin may be chosen from solvents that are known to be usable for polypeptide (protein) condensation reactions. Examples of such solvents are acid amides such as N,N-dimethylformamide, N,N-dimethylacetamide, N-methylpyrrolidone, etc.; halogenated hydrocarbons such as methylene chloride, chloroform, etc.; alcohols such as trifluoroethanol, etc.; sulfoxides such as dimethylsulfoxide, etc.; ethers such as pyridine, dioxane, tetrahydrofuran, etc.; nitriles such as acetonitrile, propionitrile, etc.; esters such as methyl acetate, ethyl acetate, etc.; and appropriate mixtures of these solvents. The reaction temperature is appropriately chosen from the range known to be applicable to polypeptide (protein) binding reactions and is usually selected in the range of approximately 20°C to 50°C. The activated amino acid derivatives are used generally in an excess of 1.5 to 4 times. The condensation is examined using the ninhydrin reaction; when the condensation is insufficient, the condensation can be completed by repeating the condensation reaction without removal of the protecting groups. When the condensation is yet insufficient even after repeating the reaction, unreacted amino acids are acetylated with acetic anhydride or acetylimidazole to cancel any possible adverse affect on the subsequent reaction.

[0037] Examples of the protecting groups used to protect the starting amino groups include Z, Boc, t-pentyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthaloyl, formyl, 2-nitrophenylsulphenyl, diphenylphosphinothioyl, Fmoc, etc.

[0038] A carboxyl group can be protected by, e.g., alkyl esterification (in the form of linear, branched or cyclic alkyl esters of the alkyl moiety such as methyl, ethyl, propyl, butyl, t-butyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, 2-adamantyl, etc.), aralkyl esterification (e.g., esterification in the form of benzyl ester, 4-nitrobenzyl ester, 4-methoxybenzyl ester, 4-chlorobenzyl ester, benzhydryl ester, etc.), phenacyl esterification, benzyloxycarbonyl hydrazidation, t-butoxycarbonyl hydrazidation, trityl hydrazidation, or the like.

[0039] The hydroxyl group of serine can be protected through, for example, its esterification or etherification. Examples of groups appropriately used for the esterification include a lower (C_{1-6}) alkanoyl group, such as acetyl group, an aroyl group such as benzoyl group, and a group derived from carbonic acid such as benzyloxycarbonyl group and ethoxycarbonyl group. Examples of a group appropriately used for the etherification include benzyl group, tetrahydropyranyl group, t-butyl group, etc.

[0040] Examples of groups for protecting the phenolic hydroxyl group of tyrosine include Bzl, Cl_2-Bzl, 2-nitrobenzyl, Br-Z, t-butyl, etc.

[0041] Examples of groups used to protect the imidazole moiety of histidine include Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, Fmoc, etc.

[0042] Examples of the activated carboxyl groups in the starting materials include the corresponding acid anhydrides, azides, activated esters [esters with alcohols (e.g., pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, p-nitrophenol, HONB, N-hydroxysuccimide, N-hydroxyphthalimide, HOBt)]. As the activated amino acids in which the amino groups are activated in the starting material, the corresponding phosphoric amides are employed.

[0043] To eliminate (split off) the protecting groups, there are used catalytic reduction under hydrogen gas flow in the presence of a catalyst such as Pd-black or Pd-carbon; an acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid or trifluoroacetic acid, or a mixture solution of these acids; a treatment with a base such as diisopropylethylamine, triethylamine, piperidine or piperazine; and reduction with sodium in liquid ammonia. The elimination of the protecting group by the acid treatment described above is carried out generally at a temperature of approximately -20°C to 40°C. In the acid treatment, it is efficient to add a cation scavenger such as anisole, phenol, thioanisole, m-cresol, p-cresol, dimethylsulfide, 1,4-butanedithiol or 1,2-ethanedithiol. Furthermore, 2,4-dinitrophenyl group known as the protecting group for the imidazole of histidine is removed by a treatment with thiophenol. Formyl group used as the protecting group of the indole of tryptophan is eliminated by the aforesaid acid treatment in the presence of 1,2-ethanedithiol or 1,4-butanedithiol, as well as by a treatment with an alkali such as a dilute sodium hydroxide solution and dilute ammonia.

[0044] Protection of functional groups that should not be involved in the reaction of the starting materials, protecting groups, elimination of the protecting groups and activation of functional groups involved in the reaction may be appropriately selected from publicly known groups and publicly known means.

[0045] In another method for obtaining the amides of the polypeptide, for example, the α-carboxyl group of the carboxy terminal amino acid is first protected by amidation; the peptide (polypeptide) chain is then extended to the amino group side to a desired length. Thereafter, a polypeptide in which only the protecting group of the N-terminal α-amino group of the peptide chain has been eliminated from the polypeptide and a polypeptide in which only the protecting group of the C-terminal carboxyl group has been eliminated are manufactured. The two polypeptides are condensed in a mixture of the solvents described above. The details of the condensation reaction are the same as described above. After the protected polypeptide obtained by the condensation is purified, all the protecting groups are eliminated by the method described above to give the desired crude polypeptide. This crude polypeptide is purified by various known purification means. Lyophilization of the major fraction gives the amide of the desired polypeptide.

[0046] To prepare the esterified polypeptide of the present invention, for example, the α-carboxyl group of the carboxy terminal amino acid is condensed with a desired alcohol to prepare the amino acid ester, which is followed by procedure similar to the preparation of the amidated polypeptide above to give the desired esterified polypeptide or partial peptide.

[0047] The polypeptide of the present invention or its salts can be manufactured by publicly known methods for peptide synthesis. For the methods for peptide synthesis, for example, either solid phase synthesis or liquid phase synthesis may be used. That is, the partial peptide or amino acids that can constitute the objective peptide of the present invention are condensed with the remaining part of the partial peptide. Where the product contains protecting groups, these protecting groups are removed to give the desired peptide. Publicly known methods for condensation and elimination of the protecting groups are described in (1) - (5) below.

(1) M. Bodanszky & M.A. Ondetti: Peptide Synthesis, Interscience Publishers, New York (1966)

(2) Schroeder & Luebke: The Peptide, Academic Press, New York (1965)

(3) Nobuo Izumiya, et al.: *Peptide Gosei-no-Kiso to Jikken* (Basics and experiments of peptide synthesis), published by Maruzen Co. (1975)

(4) Haruaki Yajima & Shunpei Sakakibara: *Seikagaku Jikken Koza* (Biochemical Experiment) 1, *Tanpakushitsu no Kagaku* (Chemistry of Proteins) IV, 205 (1977)

(5) Haruaki Yajima ed.: *Zoku Iyakuhin no Kaihatsu* (A sequel to Development of Pharmaceuticals), Vol. 14, Peptide Synthesis, published by Hirokawa Shoten

[0048] After completion of the reaction, the product may be purified and isolated by a combination of conventional purification methods such as solvent extraction, distillation, column chromatography, liquid chromatography and recrystallization to give the polypeptide of the present invention. When the polypeptide obtained by the above methods is in a free form, the polypeptide can be converted into an appropriate salt by a publicly known method; when the polypeptide is obtained in a salt form, it can be converted into a free form or a different salt form by a publicly known method.

[0049] The DNA encoding the polypeptide of the present invention may be any DNA so long as it comprises the base sequence encoding the polypeptide of the present invention described above. Such a DNA may also be any one of genomic DNA, cDNA derived from the cells or tissues described above and synthetic DNA.

[0050] The vector to be used for the library may be any of bacteriophage, plasmid, cosmid, phagemid and the like. In addition, the DNA can be amplified by reverse transcriptase polymerase chain reaction (hereinafter abbreviated as RT-PCR) with total RNA or mRNA fraction prepared from the above-described cells or tissues.

[0051] Specifically, the DNA encoding the polypeptide of the present invention may be any one of, for example, DNA comprising the base sequence represented by SEQ ID NO:23, or any DNA having a base sequence hybridizable to the base sequence represented by SEQ ID NO:23 under high stringent conditions and encoding a polypeptide which has the activities substantially equivalent to those of the polypeptide of the present invention (e.g., immunogenicity, etc.).

[0052] As the DNA comprising the base sequence represented by SEQ ID NO:23, there is employed a DNA comprising the base sequence represented by SEQ ID NO:24, or the like.

[0053] Specific examples of the DNA that is hybridizable to the base sequence represented by SEQ ID NO:23 under high stringent conditions include DNA comprising at least about 60% homology, preferably at least about 70% homology, and most preferably at least about 80% homology, to the base sequence represented by SEQ ID NO:23, and the like.

[0054] Also, specific examples of the DNA encoding the polypeptide of the present invention include DNA comprising the base sequence represented by SEQ ID NO:25, DNA having a base sequence, which is hybridizable to the base sequence represented by SEQ ID NO:25 under high stringent conditions, encodes a polypeptide which has the activities substantially equivalent to those of the polypeptide of the present invention (e.g., immunogenicity, etc.) and has a property substantially equivalent to that of the polypeptide of the present invention.

[0055] As the DNA that is hybridizable to the base sequence represented by SEQ ID NO:25 under high stringent conditions, there is employed DNA comprising at least about 60% homology, preferably at least about 70% homology, and most preferably at least about 80% homology, to the base sequence represented by SEQ ID NO:25, and the like. Specifically, DNA comprising the base sequence represented by SEQ ID NO:10, or the like is employed.

[0056] Also, specific examples of the DNA hybridizable to the base sequence represented by SEQ ID NO:23 under high stringent conditions, include DNA comprising a base sequence shown by SEQ ID NO:48, or DNA encoding a polypeptide, which comprises a base sequence hybridizable to the base sequence represented by SEQ ID NO:48 under high stringent conditions, bears DNA or the like encoding a polypeptide which has the activities substantially equivalent to those of the polypeptide of the present invention (e.g., immunogenicity, etc.) and has a property substantially equivalent to that of the polypeptide of the present invention, and the like.

[0057] As the DNA that is hybridizable to the base sequence represented by SEQ ID NO:48 under high stringent conditions, there is employed DNA having at least about 60% homology, preferably at least about 70% homology, and most preferably at least about 80% homology, to the base sequence represented by SEQ ID NO:48, and the like. Specifically, DNA containing the base sequence represented by SEQ ID NO:41 or SEQ ID NO:46, or the like is employed.

[0058] The hybridization can be carried out by publicly known methods or by a modification thereof, for example, according to the method described in Molecular Cloning, 2nd. (J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989). A commercially available library may also be used according to the instructions of the attached manufacturer's protocol. The hybridization can be carried out preferably under high stringent conditions.

[0059] The high stringent conditions are, for example, those in a sodium concentration at about 19 mM to about 40 mM, preferably about 19 mM to about 20 mM at a temperature of about 50°C to about 70°C, preferably about 60°C to about 65°C.

[0060] For the DNA encoding the polypeptide of the present invention containing the amino acid sequence represented by SEQ ID NO:24, there may be employed DNA having the base sequence represented by SEQ ID NO:23, etc. and, DNA having the base sequence represented by SEQ ID NO:4 may be used for the DNA encoding the polypeptide of the present invention having the amino acid sequence represented by SEQ ID NO:6. For the DNA encoding the polypeptide having the amino acid sequence represented by SEQ ID NO:26, DNA having the base sequence

represented by SEQ ID NO:25 may be employed and, DNA having the base sequence represented by SEQ ID NO: 10 may be used as the DNA encoding the polypeptide having the amino acid sequence represented by SEQ ID NO: 12. As the DNA encoding the polypeptide having the amino acid sequence represented by SEQ ID NO:49, there may be employed DNA having the base sequence represented by SEQ ID NO:48 and, DNA having the base sequence represented by SEQ ID NO:46 may be used for the DNA encoding the polypeptide having the amino acid sequence represented by SEQ ID NO:47.

[0061] For cloning of the DNA that entirely encodes the polypeptide of the present invention, the DNA may be either amplified by publicly known PCR using synthetic DNA primers containing a part of the base sequence of the polypeptide of the present invention, or the DNA inserted into an appropriate vector can be selected by hybridization with a labeled DNA fragment or synthetic DNA that encodes a part or entire region of the polypeptide of the present invention. The hybridization can be carried out, for example, according to the method described in Molecular Cloning, 2nd (J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989). The hybridization may also be performed using commercially available library in accordance with the protocol described in the attached instructions.

[0062] Substitution of the base sequence of DNA can be effected by PCR or publicly known methods such as the Gapped duplex method or the Kunkel method, or its modification using a publicly known kit available as Mutan™-G or Mutan™-K (both manufactured by Takara Shuzo Co., Ltd., trademark).

[0063] The cloned DNA encoding the polypeptide can be used as it is, depending upon purpose or, if desired, after digestion with a restriction enzyme or after addition of a linker thereto. The DNA may contain ATG as a translation initiation codon at the 5' end thereof and TAA, TGA or TAG as a translation termination codon at the 3' end thereof. These translation initiation and termination codons may also be added by using an appropriate synthetic DNA adapter.

[0064] The expression vector of the polypeptide of the present invention can be manufactured, for example, by (a) excising the desired DNA fragment from the DNA encoding the polypeptide of the present invention, (b) and then ligating the DNA fragment with an appropriate expression vector downstream a promoter in the vector.

[0065] Examples of the vector include plasmids derived form E. coli (e.g., pBR322, pBR325, pUC12, pUC13), plasmids derived from Bacillus subtilis (e.g., pUB110, pTP5, pC194), plasmids derived from yeast (e.g., pSH19, pSH15), bacteriophages such as λ phage, etc., animal viruses such as retrovirus, vaccinia virus, baculovirus, etc. as well as pA1-11, pXT1, pRc/CMV, pRc/RSV, pcDNAI/Neo, etc.

[0066] The promoter used in the present invention may be any promoter if it matches well with a host to be used for gene expression. In the case of using animal cells as the host, examples of the promoter include SRα promoter, SV40 promoter, LTR promoter, CMV promoter, HSV-TK promoter, β-actin, etc.

[0067] Among them, CMV (cytomegalovirus) promoter, SRα promoter or the like is preferably used. Where the host is bacteria of the genus Escherichia, preferred examples of the promoter include trp promoter, lac promoter, recA promoter, λPL promoter, lpp promoter, T7 promoter, etc. In the case of using bacteria of the genus Bacillus as the host, preferred example of the promoter are SPO1 promoter, SPO2 promoter, penP promoter, etc. When yeast is used as the host, preferred examples of the promoter are PHO5 promoter, PGK promoter, GAP promoter, ADH promoter, etc. When insect cells are used as the host, preferred examples of the promoter include polyhedrin prompter, P10 promoter, etc.

[0068] In addition to the foregoing examples, the expression vector may further optionally contain an enhancer, a splicing signal, a poly A addition signal, a selection marker, SV40 replication origin (hereinafter sometimes abbreviated as SV40ori) etc. Examples of the selection marker include dihydrofolate reductase (hereinafter sometimes abbreviated as dhfr) gene [methotrexate (MTX) resistance], ampicillin resistant gene (hereinafter sometimes abbreviated as Amp^r), neomycin resistant gene (hereinafter sometimes abbreviated as Neo^r, Geneticin resistance), etc. In particular, when dhfr gene is used as the selection marker together with dhfr gene deficient Chinese hamster cells, recombinant somatic cells can also be selected on thymidine free media.

[0069] If necessary and desired, a signal sequence that matches with a host is added to the N-terminus of the polypeptide of the present invention. Examples of the signal sequence that can be used are Pho A signal sequence, OmpA signal sequence, etc. in the case of using bacteria of the genus Escherichia as the host; α-amylase signal sequence, subtilisin signal sequence, etc. in the case of using bacteria of the genus Bacillus as the host; MFα signal sequence, SUC2 signal sequence, etc. in the case of using yeast as the host; and insulin signal sequence, α-interferon signal sequence, antibody molecule signal sequence, etc. in the case of using animal cells as the host, respectively.

[0070] Using the vector comprising the DNA encoding the polypeptide of the present invention thus constructed, transformants can be manufactured.

[0071] Examples of the host, which may be employed, are bacteria belonging to the genus Escherichia, bacteria belonging to the genus Bacillus, yeast, insect cells, insects and animal cells, etc.

[0072] Specific examples of the bacteria belonging to the genus Escherichia include Escherichia coli K12 DH1 (Proc. Natl. Acad. Sci. U.S.A., 60, 160 (1968)), JM103 (Nucleic Acids Research, 9, 309 (1981)), JA221 (Journal of Molecular Biology, 120, 517 (1978)), HB101 (Journal of Molecular Biology, 41, 459 (1969)), C600 (Genetics, 39, 440 (1954)), etc.

[0073] Examples of the bacteria belonging to the genus Bacillus include Bacillus subtilis MI114 (Gene, 24, 255

(1983)), 207-21 (Journal of Biochemistry, 95, 87 (1984)), etc.

[0074] Examples of yeast include Saccharomyces cerevisiae AH22, AH22R⁻, NA87-11A. DKD-5D, 20B-12, Schizosaccharomyces pombe NCYC1913, NCYC2036, Pichia pastoris KM71, etc.

[0075] Examples of insect cells include, for the virus AcNPV, Spodoptera frugiperda cells (Sf cells), MG1 cells derived from mid-intestine of Trichoplusia ni, High Five™ cells derived from egg of Trichoplusia ni, cells derived from Mamestra brassicae, cells derived from Estigmena acrea, etc.; and for the virus BmNPV, Bombyx mori N cells (BmN cells), etc. is used. Examples of the Sf cells which can be used are Sf9 cells (ATCC CRL1711) and Sf21 cells (both cells are described in Vaughn, J. L. et al., In Vivo, 13, 213-217 (1977).

[0076] As the insect, for example, a larva of Bombyx mori can be used (Maeda et al., Nature, 315, 592 (1985)).

[0077] Examples of animal cells include monkey cell COS-7, Vero, Chinese hamster cells CHO (hereinafter referred to as CHO cells), dhfr gene deficient Chinese hamster cells CHO (hereinafter simply referred to as CHO(dhfr⁻) cells), mouse L cells, mouse AtT-20, mouse myeloma cells, rat GH 3, human FL cells, etc.

[0078] Bacteria belonging to the genus Escherichia can be transformed, for example, by the method described in Proc. Natl. Acad. Sci. U.S.A., 69, 2110 (1972) or Gene, 17, 107 (1982).

[0079] Bacteria belonging to the genus Bacillus can be transformed, for example, by the method described in Molecular & General Genetics, 168, 111 (1979).

[0080] Yeast can be transformed, for example, by the method described in Methods in Enzymology, 194, 182-187 (1991) or Proc. Natl. Acad. Sci. U.S.A., 75, 1929 (1978).

[0081] Insect cells or insects can be transformed, for example, according to the method described in Bio/Technology, 6, 47-55(1988).

[0082] Animal cells can be transformed, for example, according to the method described in *Saibo Kogaku* (Cell Engineering), extra issue 8, *Shin Saibo Kogaku Jikken Protocol* (New Cell Engineering Experimental Protocol), 263-267 (1995), published by Shujunsha, or Virology, 52, 456 (1973).

[0083] Thus, the transformant transformed with the expression vector comprising the DNA encoding the polypeptide can be obtained.

[0084] Where the host is bacteria belonging to the genus Escherichia or the genus Bacillus, the transformant can be appropriately incubated in a liquid medium which contains materials required for growth of the transformant such as carbon sources, nitrogen sources, inorganic materials, etc. Examples of the carbon sources include glucose, dextrin, soluble starch, sucrose, etc. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrate salts, corn steep liquor, peptone, casein, yeast extract, meat extract, soybean cake, potato extract, etc. Examples of the inorganic materials are calcium chloride, sodium dihydrogenphosphate, magnesium chloride, etc. In addition, yeast extract, vitamins, growth promoting factors, etc. may also be added to the medium. Preferably, pH of the medium is adjusted to about 5 to about 8.

[0085] A preferred example of the medium for incubation of the bacteria belonging to the genus Escherichia is M9 medium supplemented with glucose and Casamino acids (Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972). If necessary and desired, a chemical such as 3β-indolylacrylic acid can be added to the medium thereby to function the promoter efficiently.

[0086] Where the bacteria belonging to the genus Escherichia are used as the host, the transformant is usually cultivated at about 15°C to about 43°C for about 3 hours to about 24 hours. If necessary and desired, the culture may be aerated or agitated.

[0087] Where the bacteria belonging to the genus Bacillus are used as the host, the transformant is cultivated generally at about 30°C to about 40°C for about 6 hours to about 24 hours. If necessary and desired, the culture can be aerated or agitated.

[0088] Where yeast is used as the host, the transformant is cultivated, for example, in Burkholder's minimal medium [Bostian, K. L. et al., Proc. Natl. Acad. Sci. U.S.A., 77, 4505 (1980)] or in SD medium supplemented with 0.5% Casamino acids [Bitter, G. A. et al., Proc. Natl. Acad. Sci. U.S.A., 81, 5330 (1984)]. Preferably, pH of the medium is adjusted to about 5 to about 8. In general, the transformant is cultivated at about 20°C to about 35°C for about 24 hours to about 72 hours. If necessary and desired, the culture can be aerated or agitated.

[0089] Where insect cells or insects are used as the host, the transformant is cultivated in, for example, Grace's Insect Medium (Grace, T. C. C., Nature, 195, 788 (1962)) to which an appropriate additive such as immobilized 10% bovine serum is added. Preferably, pH of the medium is adjusted to about 6.2 to about 6.4. Normally, the transformant is cultivated at about 27°C for about 3 days to about 5 days and, if necessary and desired, the culture can be aerated or agitated.

[0090] Where animal cells are employed as the host, the transformant is cultivated in, for example, MEM medium containing about 5% to about 20% fetal calf serum [Science, 122, 501 (1952)], DMEM medium [Virology, 8, 396 (1959)], RPMI 1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], 199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)], etc. Preferably, pH of the medium is adjusted to about 6 to about 8. The transformant is usually cultivated at about 30°C to about 40°C for about 15 hours to about 60 hours and, if

necessary and desired, the culture can be aerated or agitated.

[0091] As described above, the polypeptide of the present invention can be produced intracellularly or extracellularly in the transformant.

[0092] The polypeptide of the present invention can be separated and purified from the culture described above by the following procedures.

[0093] When the polypeptide of the present invention is extracted from the culture or cells, after cultivation the cell or transformant is collected by a publicly known method and suspended in a appropriate buffer. The cell or transformant is then disrupted by publicly known methods such as ultrasonication, a treatment with lysozyme and/or freeze-thaw, etc. followed by centrifugation or filtration. Thus, the crude extract of the polypeptide can be obtained. The buffer used for the procedures may contain a protein modifier such as urea or guanidine hydrochloride, or a surfactant such as Triton X-100™. etc. When the polypeptide is secreted in the culture broth, after completion of the cultivation the supernatant can be separated from the cell or transformant to collect the supernatant by a publicly known method.

[0094] The supernatant or the polypeptide contained in the extract thus obtained can be purified by appropriately combining the publicly known methods for separation and purification. Such publicly known methods for separation and purification include a method utilizing difference in solubility such as salting out, solvent precipitation, etc.; a method mainly utilizing difference in molecular weight such as dialysis, ultrafiltration, gel filtration, SDS-polyacrylamide gel electrophoresis, etc.; a method utilizing difference in electric charge such as ion exchange chromatography, etc.; a method utilizing difference in specific affinity such as affinity chromatography, etc.; a method utilizing difference in hydrophobicity such as reverse phase high performance liquid chromatography, etc.; a method utilizing difference in isoelectric point such as isoelectrofocusing electrophoresis; and the like.

[0095] When the polypeptide thus obtained is in a free form, it can be converted into the salt by publicly known methods or modifications thereof. On the other hand, when the polypeptide is obtained in the form of a salt, it can be converted into the free form or in the form of a different salt by publicly known methods or modifications thereof.

[0096] The polypeptide produced by the recombinant can be treated, prior to or after the purification, with an appropriate protein modifying enzyme so that the polypeptide can be appropriately modified to remove a part of the polypeptide. Examples of these enzymes include trypsin, chymotrypsin, arginyl endopeptidase, protein kinase, glycosidase and the like.

[0097] The activity of the thus produced polypeptide of the present invention or salts thereof can be determined by an enzyme immunoassay using a specific antibody, Western blot analysis, etc.

[0098] Antibodies to the polypeptide of the present invention or salts thereof may be any of polyclonal antibodies and monoclonal antibodies, as long as they are capable of recognizing the polypeptide of the present invention or salts thereof.

[0099] The antibodies to the polypeptide of the present invention or salts thereof may be manufactured by publicly known methods for manufacturing antibodies or antisera, using as antigens the polypeptide of the present invention.

[Preparation of monoclonal antibody]

(a) Preparation of monoclonal antibody-producing cells

[0100] The polypeptide of the present invention or its salt is administered to warm-blooded animals either solely or together with carriers or diluents to the site, in which the production of antibody is possible by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvants or incomplete Freund's adjuvants may be administered. The administration is usually carried out once every two to six weeks and approximately two to ten times in total. Examples of the applicable warm-blooded animals are monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats, chickens and the like, with the use of mice and rats being preferred.

[0101] In the preparation of monoclonal antibody-producing cells, a warm-blooded animal, e.g., mice, immunized with an antigen wherein the antibody titer is recognized-is selected, then spleen or lymph node is collected after two to five days from the final immunization and antibody-producing cells contained therein are fused with myeloma cells from homozoic or heterozoic animal to give monoclonal antibody-producing hybridomas. Measurement of the antibody titer in antisera may be carried out, for example, by reacting a labeled form of the polypeptide, which will be described later, with the antiserum followed by assaying the binding activity of the labeling agent bound to the antibody. The fusion may be carried out, for example, using the known method by Koehler and Milstein [Nature, 256, 495, (1975)]. Examples of the fusion accelerator are polyethylene glycol (PEG), Sendai virus, etc., of which PEG is preferably employed.

[0102] Examples of the myeloma cells are those collected from warm-blooded animals such as NS-1, P3U1, SP2/0, AP-1, etc. In particular, P3U1 is preferably employed. A preferred ratio of the count of the antibody-producing cells used (spleen cells) to the count of myeloma cells is within a range of approximately 1:1 to 20:1. When PEG (preferably, PEG 1000 to PEG 6000) is added in a concentration of approximately 10 to 80% followed by incubating at 20 to 40°C,

preferably at 30 to 37°C for about 1 to about 10 minutes, an efficient cell fusion can be carried out.

[0103] Various methods can be used for screening of monoclonal antibody-producing hybridomas. Examples of such methods include a method which comprises adding the supernatant of hybridoma to a solid phase (e.g., microplate) adsorbed with, e.g., a polypeptide antigen directly or together with a carrier, adding an anti-immunoglobulin antibody (when mouse cells are used for the cell fusion, anti-mouse immunoglobulin antibody is used) labeled with a radioactive substance or an enzyme, or adding Protein A and detecting the monoclonal antibody bound to the solid phase, and a method which comprises adding the supernatant of hybridoma to a solid phase adsorbed with an anti-immunoglobulin antibody or Protein A, adding the polypeptide labeled with a radioactive substance or an enzyme and detecting the monoclonal antibody bound to the solid phase.

[0104] The monoclonal antibody can be selected according to publicly known methods or their modifications. In general, the selection can be effected in a medium for animal cells supplemented with HAT (hypoxanthine, aminopterin and thymidine). Any selection and growth medium can be employed as far as the hybridoma can grow there. For example, RPMI 1640 medium containing about 1% to about 20%, preferably about 10% to about 20% fetal calf serum, GIT medium (Wako Pure Chemical Industries, Ltd.) containing 1% to 10% fetal calf serum, a serum free medium for cultivation of a hybridoma (SFM-101, Nissui Seiyaku Co., Ltd.) and the like can be used for the selection and growth medium. The cultivation is carried out generally at 20°C to 40°C, preferably at 37°C, for about 5 days to about 3 weeks, preferably 1 to 2 weeks, normally in 5% CO_2. The antibody titer of the culture supernatant of a hybridoma can be determined as in the assay for the antibody titer in antisera described above.

(b) Purification of monoclonal antibody

[0105] Separation and purification of a monoclonal antibody can be carried out by publicly known methods, such as separation and purification of immunoglobulins [e.g., salting-out, alcohol precipitation, isoelectric point precipitation, electrophoresis, adsorption and desorption with ion exchangers (e.g., DEAE), ultracentrifugation, gel filtration, or a specific purification method which comprises collecting only an antibody with an activated adsorbent such as an antigen-binding solid phase, Protein A or Protein G and dissociating the binding to obtain the antibody].

[Preparation of polyclonal antibody]

[0106] The polyclonal antibody of the present invention can be manufactured by publicly known methods or modifications thereof. For example, a warm-blooded animal is immunized with an immunogen (polypeptide antigen) per se, or a complex of immunogen and a carrier protein is formed and a warm-blooded animal is immunized with the complex in a manner similar to the method described above for the manufacture of monoclonal antibodies. The product containing the antibody to the polypeptide of the present invention or its salt is collected from the immunized animal followed by separation and purification of the antibody.

[0107] In the complex of immunogen and carrier protein used to immunize a warm-blooded animal, the type of carrier protein and the mixing ratio of carrier to hapten may be any type and in any ratio, as long as the antibody is efficiently produced to the hapten immunized by crosslinking to the carrier. For example, bovine serum albumin, bovine thyroglobulin, hemocyanin or the like is coupled to hapten in a carrier-to-hapten weight ratio of approximately 0.1 to 20, preferably about 1 to about 5.

[0108] A variety of condensation agents can be used for the coupling of carrier to hapten. Glutaraldehyde, carbodiimide, maleimide activated ester, activated ester reagents containing thiol group or dithiopyridyl group and the like are used for the coupling.

[0109] The condensation product is administered to warm-blooded animals either solely or together with carriers or diluents to the site that can produce the antibody by the administration. In order to potentiate the antibody productivity upon the administration, complete Freund's adjuvant or incomplete Freund's adjuvant may be administered. The administration is usually made once every about 2 to about 6 weeks and about 3 to about 10 times in total.

[0110] The polyclonal antibody can be collected from the blood, ascites, etc., preferably from the blood of warm-blooded animal immunized by the method described above.

[0111] The polyclonal antibody titer in antiserum can be assayed by the same procedure as that for the determination of serum antibody titer described above. The separation and purification of the polyclonal antibody can be carried out, following the method for the separation and purification of immunoglobulins performed as in the separation and purification of monoclonal antibodies described hereinabove.

[0112] The antisense DNA having a complementary or substantially complementary base sequence to the DNA encoding the polypeptide of the present invention can be any antisense DNA so long as it possesses a base sequence a complementary or substantially complementary base sequence to that of the DNA of the present invention and capable of suppressing expression of the DNA.

[0113] The base sequence substantially complementary to the DNA of the present invention may, for example, be

a base sequence having at least about 70% homology, preferably at least about 80% homology, more preferably at least about 90% homology and most preferably at least about 95% homology, to the full-length base sequence or partial base sequence of the base sequence complementary to the DNA of the present invention (i.e., complementary strand to the DNA of the present invention). Particularly in the entire base sequence of the complementary strand to the DNA of the present invention, an antisense DNA having at least about 70% homology, preferably at least about 80% homology, more preferably at least about 90% homology and most preferably at least about 95% homology, to the complementary strand of the base sequence which encodes the N-terminal region of the polypeptide of the present invention (e.g., the base sequence around the initiation codon). These antisense DNAs can be synthesized using a publicly known DNA synthesizer, etc.

[0114] In the case that the polypeptide of the present invention has a signal peptide, it is efficiently secreted extracellularly to exhibit as a humoral factor important biological activities such as signal transduction, self defense, etc.

[0115] Hereinafter the uses of the polypeptide of the present invention or salts thereof (hereinafter sometimes merely referred to as the polypeptide of the present invention); DNA encoding the polypeptide of the present invention (hereinafter sometimes merely referred to as the DNA of the present invention), antibodies to the polypeptide of the present invention, or salts thereof (hereinafter sometimes merely referred to as the antibody of the present invention) and the antisense DNA.

(1) Since the polypeptide of the present invention is expressed specifically to cartilage tissues, the polypeptide can be used as a tissue marker. That is, the polypeptide is useful as a marker for detecting the differentiation, pathological conditions, metastasis of cancer, etc. The polypeptide is also applicable to fractionation of the corresponding receptors, ligands, bound polypeptides, etc. Furthermore, the polypeptide may be formed into a panel for publicly known high through-put screening, which can be utilized for exploring biological activities. In addition, through the chromosomal mapping, the polypeptide is also available for studies on genetic diseases.

(2) Therapeutic/prophylactic agent for the diseases with which the polypeptide of the present invention of the present invention is associated

[0116] Since the polypeptide of the present invention is present in vivo (especially in the cartilage tissue) as a humoral factor and has a function to suppress differentiation of cartilage, any abnormality or deficiency of the polypeptide of the present invention or the DNA of the present invention or any abnormal reduction or accentuation in the expression amount of the polypeptide or the DNA would cause a variety of diseases.

[0117] When the DNA, etc. of the present invention is deficient or its expression amount is abnormally reduced, such would cause various diseases including bone and joint diseases, e.g., arthritis deformans, chronic articular rheumatism, marble stone disease, etc.; or pathological angiogenesis, etc.

[0118] Therefore, the polypeptide of the present invention and the DNA of the present invention can be used as pharmaceuticals such as agents for the treatment/prevention of various diseases such as bone and joint diseases, e.g., arthritis deformans, chronic articular rheumatism, marble stone disease, etc.; or pathological angiogenesis, etc.

[0119] When a patient has a reduced level of, or deficient of the polypeptide of the present invention in his or her body so that signal transduction in cells does not work sufficiently or normally, the DNA of the present invention can provide its role sufficiently or properly for the patient, (a) by administering the DNA of the present invention to the patient to express the polypeptide of the present invention in vivo, (b) by inserting the DNA of the present invention into a cell, expressing the polypeptide of the present invention and then transplanting the cell to the patient, or (c) by administering the polypeptide of the present invention to the patient.

[0120] Where the DNA of the present invention is used as the prophylactic/therapeutic agents described above, the DNA per se can be administered directly to human or other warm-blooded animal; alternatively, the DNA can be inserted into an appropriate vector such as retrovirus vector, adenovirus vector, adenovirus-associated virus vector, etc. and then administered to human or other warm-blooded animal in a conventional manner. The DNA of the present invention may also be administered as naked DNA, or with adjuvants to assist its uptake by gene gun or through a catheter such as a catheter with a hydrogel.

[0121] Where the polypeptide of the present invention is used as the aforesaid therapeutic/prophylactic agents, the polypeptide is advantageously used on a purified level of at least 90%, preferably at least 95%, more preferably at least 98% and most preferably at least 99%.

[0122] The polypeptide of the present invention can be used orally, for example, in the form of tablets which may be sugar coated if necessary and desired, capsules, elixirs, microcapsules etc., or parenterally in the form of injectable preparations as a sterile solution and a suspension in water or with other pharmaceutically acceptable liquid. These preparations can be manufactured by mixing the polypeptide of the present invention with a physiologically acceptable known carrier, a flavoring agent, an excipient, a vehicle, an antiseptic agent, a stabilizer, a binder, etc. in a unit dosage form required in a generally accepted manner that is applied to making pharmaceutical preparations. The active ingredient in the preparation is controlled in such a dose that an appropriate dose is obtained within the specified range

given.

[0123] Additives miscible with tablets, capsules, etc. include a binder such as gelatin, corn starch, tragacanth and gum arabic, an excipient such as crystalline cellulose, a swelling agent such as corn starch, gelatin and alginic acid, a lubricant such as magnesium stearate, a sweetening agent such as sucrose, lactose and saccharin, and a flavoring agent such as peppermint, akamono oil and cherry. When the unit dosage is in the form of capsules, liquid carriers such as oils and fats may further be used together with the additives described above. A sterile composition for injection may be formulated according to a conventional manner used to make pharmaceutical compositions, e.g., by dissolving or suspending the active ingredients in a vehicle such as water for injection with a naturally occurring vegetable oil such as sesame oil and coconut oil, etc. to prepare the pharmaceutical composition.

[0124] Examples of an aqueous medium for injection include physiological saline and an isotonic solution containing glucose and other auxiliary agents (e.g., D-sorbitol, D-mannitol, sodium chloride, etc.) and may be used in combination with an appropriate dissolution aid such as an alcohol (e.g., ethanol or the like), a polyalcohol (e.g., propylene glycol and polyethylene glycol), a nonionic surfactant (e.g., polysorbate 80™, HCO-50, etc.) and the like. Examples of the oily medium include sesame oil and soybean oil, which may also be used in combination with a dissolution aid such as benzyl benzoate and benzyl alcohol. The prophylactic/therapeutic agent described above may further be formulated with a buffer (e.g., phosphate buffer, sodium acetate buffer, etc.), a soothing agent (e,g., benzalkonium chloride, procaine hydrochloride, etc.), a stabilizer (e.g., human serum albumin, polyethylene glycol, etc.), a preservative (e.g., benzyl alcohol, phenol, etc.), an antioxidant, etc. The thus-prepared liquid for injection is normally filled in an appropriate ampoule.

[0125] The vector in which the DNA of the present invention is inserted may also be prepared into pharmaceutical preparations in a manner similar to the procedures above. Such preparations are generally used parenterally.

[0126] Since the thus obtained pharmaceutical preparation is safe and low toxic, the preparation can be administered to human or other warm-blooded animal (e.g., rat, mouse, guinea pig, rabbit, chicken, sheep, swine, bovine, horse, cat, dog, monkey, etc.).

[0127] The dose of the polypeptide of the present invention varies depending on target disease, subject to be administered, route for administration, etc.; for example, in oral administration for the treatment of bone and joint disease, the polypeptide of the present invention is normally administered in a dose of about 1 mg to about 1000 mg, preferably about 10 to about 500 mg, and more preferably about 10 to about 200 mg per day for adult (as 60 kg body weight). In parenteral administration, the single dose of the polypeptide of the present invention varies depending on subject to be administered, target disease, etc. but it is advantageous for the treatment of bone and joint disease to administer the active ingredient intravenously at a daily dose of about 1 to about 1000 mg, preferably about 1 to about 200 mg, and more preferably about 10 to about 100 mg for adult (as 60 kg body weight). For other animal species, the corresponding dose as converted per 60 kg body weight can be administered.

(2) Screening of pharmaceutical candidate compound for disease

[0128] Since the polypeptide of the present invention is present in vivo (especially in the cartilage tissue) as a humoral factor and has the function to suppress cartilage differentiation, a compound or its salt that promotes the function of the polypeptide of the present invention can be used as pharmaceuticals for the treatment/prevention of bone and joint diseases, e.g., arthritis deformans, chronic articular rheumatism, marble stone disease, etc., or pathological angiogenesis, etc.

[0129] On the other hand, a compound or its salt that inhibits the function of the polypeptide of the present invention can be used as pharmaceuticals for the treatment/prevention of diseases caused by overproduction of the polypeptide of the present invention, for example, bone and joint diseases such as arthritis deformans, chronic articular rheumatism, osteogenesis imperfecta, oseteoporosis, bone fractures, osteonecrosis of the femoral head, chondrodysplasia, etc., or pathological angiogenesis, and the like.

[0130] Therefore, the polypeptide of the present invention is useful as reagents for screening the compound or its salt that promotes or inhibits the function of the polypeptide of the present invention.

[0131] That is, the present invention provides:

[0132] (1) a method for screening the compound or its salts that promote the function of the polypeptide of the present invention or its salts (hereinafter sometimes merely referred to as the accelerator), or the compound that inhibits the function of the polypeptide of the present invention or its salts (hereinafter sometimes merely referred to as the inhibitor), which comprises using the polypeptide of the present invention or its salts.

[0133] The kit for screening of the present invention comprises the polypeptide of the present invention or its salts.

[0134] The compound or its salts obtainable by the screening method or the screening kit of the present invention is the compound selected from, e.g., peptides, proteins, non-peptide compounds, synthetic compounds, fermentation products, cell extracts, plant extracts, animal tissue extracts, plasma, etc. and is the compound that promotes or inhibits the function of the polypeptide of the present invention.

[0135] As the salts of the compound, there may be employed similar salts to those of the polypeptide of the present invention described above.

[0136] When the compound or its salts obtainable by the screening method or the screening kit of the present invention are used as the therapeutic/prophylactic agents described above, a conventional means may be applied to making pharmaceutical preparations. For example, the compound or its salts may be prepared into tablets, capsules, elixirs, microcapsules, sterile solutions, suspensions, etc.

[0137] Since the thus obtained preparation is safe and low toxic, it can be administered orally or parenterally to human or warm-blooded animal (e.g., mouse, rat, rabbit, sheep, swine, bovine, horse, chicken, cat, dog, monkey, etc.).

[0138] The dose of the compound or salts thereof varies depending on activity, target disease, subject to be administered, method for administration, etc.; for example, when the compound that accelerates the function of the polypeptide of the present invention is orally administered for the treatment of bone and joint diseases, the dose is normally about 0.1 to about 100 mg, preferably about 1.0 to about 50 mg, more preferably about 1.0 to about 20 mg per day for adult (as 60 kg body weight). In parenteral administration, the single dose varies depending on subject to be administered, target disease, etc., but for the treatment of, e.g., bone and joint diseases, it is advantageous to administer the compound that accelerates the functions of the polypeptide of the present invention intravenously at a daily dose of about 0.01 to about 30 mg, preferably about 0.1 to about 20 mg, more preferably about 0.1 to about 10 mg for adult (as 60 kg body weight). For other animal species, the corresponding dose as converted per 60 kg weight can be administered.

[0139] Turning to the compound that inhibits the functions of the polypeptide of the present invention, when it is orally administered, the dose is normally about 0.1 to about 100 mg, preferably about 1.0 to about 50 mg, more preferably about 1.0 to about 20 mg per day for adult (as 60 kg body weight). In parenteral administration, the single dose varies depending on subject to be administered, target disease, etc. When the compound that inhibits the function of the polypeptide of the present invention is administered to adult (as 60 kg body weight) generally in the form of injection, it is advantageous to administer the compound intravenously at a daily dose of about 0.01 to about 30 mg, preferably about 0.1 to about 20 mg, more preferably about 0.1 to about 10 mg. For other animal species, the corresponding dose as converted per 60 kg weight can be administered.

(3) Quantification for the polypeptide of the present invention or its salts:

[0140] The antibody to the polypeptide of the present invention (hereinafter sometimes merely referred to as the antibody of the present invention) is capable of specifically recognizing the polypeptide of the present invention and thus, can be used for a quantification of the polypeptide of the present invention in a test sample fluid, in particular, for a quantification by sandwich immunoassay.

[0141] That is, the present invention provides:

(i) a method for quantification of the polypeptide of the present invention in a test sample fluid, which comprises competitively reacting the antibody of the present invention, a test sample fluid and a labeled form of the polypeptide of the present invention, and measuring the ratio of the labeled polypeptide of the present invention bound to said antibody; and,

(ii) a method for quantification of the polypeptide of the present invention in a test sample fluid, which comprises reacting the test sample fluid simultaneously or continuously with the antibody of the present invention immobilized on a carrier and a labeled form of the antibody of the present invention, and then measuring the activity of the labeling agent on the insoluble carrier.

[0142] The monoclonal antibody to the polypeptide of the present invention (hereinafter sometimes simply referred to as the monoclonal antibody of the present invention) may be used to quantify the polypeptide of the present invention. Moreover, the polypeptide of the present invention can be detected by means of a tissue staining as well. For these purposes, the antibody molecule per se may be used or $F(ab')_2$, Fab' or Fab fractions of the antibody molecule may also be used.

[0143] There is no particular limitation for the quantification method using the antibody of the present invention to the polypeptide of the present invention; any method is usable so far as it relates to a method in which the amount of antibody, antigen or antibody-antigen complex can be detected by a chemical or a physical means, corresponding to the amount of antigen (e.g., the amount of the polypeptide of the present invention) in a test sample fluid to be detected, and then calculated using a standard curve prepared by a standard solution containing the known amount of antigen. Advantageously used are, for example, nephrometry, competitive method, immunometric method and sandwich method; in terms of sensitivity and specificity, the sandwich method, which will be described later, is particularly preferred.

[0144] Examples of the labeling agent used in the assay method using the labeling substance are radioisotopes, enzymes, fluorescent substances and luminescent substances, etc. Examples of the radioisotope are $[^{125}I]$, $[^{131}I]$, $[^{3}H]$,

[^{14}C], etc. Preferred examples of the enzyme are those that are stable and have a high specific activity, which include β-galactosidase, β-glucosidase, alkaline phosphatase, peroxidase, malate dehydrogenase, etc. Examples of the fluorescent substance are fluorescamine, fluorescein isothiocyanate, etc. Examples of the luminescent substance are luminol, a luminol derivative, luciferin, lucigenin, etc. Furthermore, the biotin-avidin system may also be used for binding of an antibody or antigen to a labeling agent.

[0145] In the immobilization of antigens or antibodies, physical adsorption may be used. Alternatively, chemical binding that is conventionally used for immobilization of proteins or enzymes may be used as well. Examples of the carrier include insoluble polysaccharides such as agarose, dextran and cellulose; synthetic resins such as polystyrene, polyacrylamide and silicone; glass; etc.

[0146] In the sandwich method, a test sample fluid is reacted with an immobilized monoclonal antibody of the present invention (first reaction), then reacted with another labeled monoclonal antibody of the present invention (second reaction) and the activity of the labeling agent on the insoluble carrier is assayed, whereby the amount of the polypeptide of the present invention in the test sample fluid can be quantified. The first and second reactions may be carried out in a reversed order, simultaneously or sequentially with an interval. The type of the labeling agent and the method for immobilization may be the same as those described hereinabove. In the immunoassay by the sandwich method, it is not always necessary that the antibody used for the labeled antibody and for the solid phase should be one type or one species but a mixture of two or more antibodies may also be used for the purpose of improving the measurement sensitivity, etc.

[0147] In the method for assaying the polypeptide of the present invention by the sandwich method according to the present invention, preferred monoclonal antibodies of the present invention used for the first and the second reactions are antibodies, which binding sites to the polypeptide of the present invention are different from one another. Thus, the antibodies used in the first and the second reactions are those wherein, when the antibody used in the second reaction recognizes the C-terminal region of the polypeptide of the present invention or the receptor protein, the antibody recognizing the site other than the C-terminal regions, e.g., recognizing the N-terminal region, is preferably used in the first reaction.

[0148] The monoclonal antibody of the present invention may be used in an assay system other than the sandwich method, such as a competitive method, an immunometric method and a nephrometry.

[0149] In the competitive method, an antigen in a test sample fluid and a labeled antigen are competitively reacted with an antibody, then the unreacted labeled antigen (F) and the labeled antigen bound to the antibody (B) are separated (i.e., B/F separation) and the labeled amount of either B or F is measured to determine the amount of the antigen in the test sample fluid. In the reactions for such a method, there are a liquid phase method in which a soluble antibody is used as the antibody and the B/F separation is effected by polyethylene glycol while a second antibody to the antibody is used, and a solid phase method in which an immobilized antibody is used as the first antibody or a soluble antibody is used as the first antibody while an immobilized antibody is used as the second antibody.

[0150] In the immunometric method, an antigen in a test sample fluid and an immobilized antigen are competitively reacted with a given amount of a labeled antibody followed by separating the solid phase from the liquid phase; or an antigen in a test sample fluid and an excess amount of labeled antibody are reacted, then an immobilized antigen is added to bind an unreacted labeled antibody to the solid phase and the solid phase is separated from the liquid phase. Thereafter, the labeled amount of any of the phases is measured to determine the antigen amount in the test sample fluid.

[0151] In the nephrometry, the amount of insoluble sediment, which is produced as a result of the antigen-antibody reaction in a gel or in a solution, is measured. Even when the amount of an antigen in a test sample fluid is small and only a small amount of the sediment is obtained, a laser nephrometry utilizing laser scattering can be suitably used.

[0152] In applying each of those immunoassays to the assay method for the present invention, any special conditions or operations are not required to set forth. The assay system for the polypeptide of the present invention may be constructed in addition to conditions or operations conventionally used for each of the methods, taking the technical consideration of one skilled in the art into account consideration. For the details of such conventional technical means, a variety of reviews, reference books, etc. may be referred to.

[0153] Reference may be made to, for example, Hiroshi Irie (ed.): "Radioimmunoassay" (published by Kodansha, 1974); Hiroshi Irie (ed.): "Radioimmunoassay; Second Series" (published by Kodansha, 1979); Eiji Ishikawa, et al. (ed.): "Enzyme Immunoassay" (published by Igaku Shoin, 1978); Eiji Ishikawa, et al. (ed.): "Enzyme Immunoassay" (Second Edition) (published by Igaku Shoin, 1982); Eiji Ishikawa, et al. (ed.): "Enzyme Immunoassay" (Third Edition) (published by Igaku Shoin, 1987); "Methods in Enzymology" Vol. 70 (Immuochemical Techniques (Part A)); ibid., Vol. 73 (Immunochemical Techniques (Part B)); ibid., Vol. 74 (Immunochemical Techniques (Part C)); ibid., Vol. 84 (Immunochemical Techniques (Part D: Selected Immunoassays)); ibid., Vol. 92 (Immunochemical Techniques (Part E: Monoclonal Antibodies and General Immunoassay Methods)); ibid., Vol. 121 (Immunochemical Techniques (Part I: Hybridoma Technology and Monoclonal Antibodies)) (published by Academic Press); etc.)

[0154] As described above, the polypeptide of the present invention can be quantified with high sensitivity, using the

antibody of the present invention.

[0155] Furthermore, by quantifying the level of the polypeptide of the present invention using the antibody of the present invention, (1) when an increase in level of the polypeptide of the present invention is detected, it can be diagnosed that the following diseases are involved or it is highly suspected of these disease to occur in the future. Examples of such diseases are bone and joint diseases (e.g., arthritis deformans, chronic articular rheumatism, osteogenesis imperfecta, oseteoporosis, bone fractures, osteonecrosis of the femoral head, chondrodysplasia, etc.) and pathological angiogenesis (e.g., tumor angiogenesis, etc.). Also, (2) when a decrease in level of the polypeptide of the present invention is detected, it can be diagnosed that the following diseases are involved or it is highly suspected of these disease to be caused in the future. Examples of such diseases are bone and joint diseases (e.g., arthritis deformans, chronic articular rheumatism, marble stone disease, etc.) and pathological angiogenesis (e.g., tumor angiogenesis, etc.)

[0156] The antibody of the present invention can be employed for detecting the polypeptide of the present invention which may be present in a test sample fluid such as a body fluid, a tissue, etc. The antibody can also be used for the preparation of an antibody column to purify the polypeptide of the present invention, detect the polypeptide of the present invention in each fraction upon purification, and analysis of the behavior of the polypeptide of the present invention in the cells under inspection.

(4) Gene diagnostic agent

[0157] By using the DNA of the present invention, e.g., as a probe, an abnormality (gene abnormality) of the DNA or mRNA encoding the polypeptide of the present invention in human or other warm-blooded animal (e.g., rat, mouse, guinea pig, rabbit, chicken, sheep, swine, bovine, horse, cat, dog, monkey, etc.)can be detected. Therefore, the DNA of the present invention is useful as a gene diagnostic agent for the damage to the DNA or mRNA, its mutation, or its decreased expression, or increased expression or overexpression of the DNA or mRNA.

[0158] The gene diagnosis described above using the DNA of the present invention can be performed by, for example, the publicly known Northern hybridization assay or the PCR-SSCP assay (Genomics, 5, 874-879 (1989); Proceedings of the National Academy of Sciences of the United States of America, 86, 2766-2770 (1989)); a DNA micro array, etc..

[0159] In case that decreased expression or overexpression is detected by, e.g., the Northern hybridization or a DNA micro array, or mutation of the DNA is detected by the PCR-SSCP method or a DNA micro array, it can be diagnosed that the following diseases are involved or it is highly likely to suffer from these disease in the future. Examples of such diseases are bone and joint diseases (e.g., arthritis deformans, chronic articular rheumatism, marble stone disease, etc.) and pathological angiogenesis (e.g., tumor angiogenesis, etc.).

(5) Pharmaceutical comprising antisense DNA

[0160] Antisense DNA that binds complemenarily to the DNA of the present invention to inhibit expression of the DNA can be used as the agent for the treatment/prevention of diseases that are caused by in vivo overexpression of the polypeptide of the present invention or the DNA of the present invention (e.g., bone and joint diseases such as arthritis deformans, chronic articular rheumatism, osteogenesis imperfecta, oseteoporosis, bone fractures, osteonecrosis of the femoral head, chondrodysplasia, etc., or pathological angiogenesis such as tumor angiogenesis, etc.).

[0161] The antisense DNA described above can be used for the therapeutic/prophylactic agent described above, as in the therapeutic/prophylactic agent of various diseases comprising the DNA of the present invention described above.

[0162] For example, the antisense DNA is administered directly, or the antisense DNA is inserted into an appropriate vector such as retrovirus vector, adenovirus vector, adenovirus-associated virus vector, etc. followed by treating in a conventional manner. The antisense DNA may be administered as it stands, or with a physiologically acceptable carrier to assist its uptake by gene gun or through a catheter such as a catheter with a hydrogel.

[0163] In addition, the antisense DNA may also be employed as an oligonucleotide probe for diagnosis to examine the presence of the DNA of the present invention in tissues or cells and states of its expression.

(6) Pharmaceutical comprising the antibody of the present invention

[0164] The antibody of the present invention which possesses the effect to neutralize the activities of the polypeptide of the present invention can be used as pharmaceuticals for the treatment/prevention of diseases that are caused by in vivo overexpression of the polypeptide of the present invention or the DNA of the present invention (e.g., bone and joint diseases such as arthritis deformans, chronic articular rheumatism, osteogenesis imperfecta, oseteoporosis, bone fractures, osteonecrosis of the femoral head, chondrodysplasia, etc., or pathological angiogenesis such as tumor angiogenesis, etc.).

[0165] The agent comprising the antibody of the present invention for the treatment and prevention of the aforesaid

diseases may be administered orally or parenterally to human or mammal (e.g., rat, rabbit, sheep, swine, bovine, cat, dog, monkey, etc.) as a liquid preparation in its original form, or as a pharmaceutical composition in an appropriate pharmaceutical formulation. The dose varies depending on subject to be administered, target disease, conditions, route for administration, etc.; for example, when used for the treatment of bone and joint disease, the antibody of the present invention is intravenously administered normally in the dose of about 0.01 mg to about 20 mg/kg body weight, preferably about 1.0 to about 10 mg/kg body weigh, and more preferably about 0.1 to about 5 mg per day once to about 5 times a day, preferably once to about 3 times. In parenteral administration via other route and in oral administration, a dose similar to those given above can be administered. Where conditions are serious, the dose may be increased depending on the conditions.

[0166] The antibody of the present invention may be administered in itself or as an appropriate pharmaceutical composition. The pharmaceutical composition used for the administration described above contains a pharmacologically acceptable carrier with the aforesaid compounds or salts thereof, a diluent or excipient. Such a composition is provided in the preparation suitable for oral or parenteral administration.

[0167] That is, examples of the composition for oral administration include solid or liquid preparations, specifically, tablets (including dragees and film-coated tablets), pills, granules, powdery preparations, capsules (including soft capsules), syrup, emulsions, suspensions, etc. Such a composition is manufactured by publicly known methods and contains a vehicle, a diluent or an excipient conventionally used in the field of pharmaceutical preparations. Examples of the vehicle or excipient for tablets are lactose, starch, sucrose, magnesium stearate, etc.

[0168] Examples of the composition for parenteral administration that can be used are injections, suppositories, etc. and the injections include the form of intravenous, subcutaneous, transcutaneous, intramuscular and drip injections. Such injections are prepared by publicly known methods, e.g., by dissolving, suspending or emulsifying the aforesaid antibody or its salts in a sterile aqueous or oily liquid medium. For the aqueous medium for injection, for example, physiological saline and isotonic solutions containing glucose and other adjuvant, etc. are used. Appropriate dissolution aids, for example, alcohol (e.g., ethanol), polyalcohol (e.g., propylene glycol, polyethylene glycol), nonionic surfactant [e.g., polysorbate 80™, HCO-50 (polyoxyethylene (50 mol) adduct of hydrogenated castor oil)] may be used in combination. For the oily solution, for example, sesame oil, soybean oil and the like are used, and dissolution aids such as benzyl benzoate and benzyl alcohol may be used in combination. The thus-prepared liquid for injection is normally filled in an appropriate ampoule. The suppository used for rectal administration is prepared by mixing the aforesaid antibody or its salts with conventional suppository base.

[0169] The oral or parenteral pharmaceutical composition described above is advantageously prepared in a unit dosage form suitable for the dose of the active ingredient. Examples of such unit dosage form include tablets, pills, capsules, injections (ampoules), suppositories, etc. It is preferred that the antibody described above is contained generally in a dose of about 5 to about 500 mg per unit dosage form, about 5 to about 100 mg especially for injections and about 10 to about 250 mg for other preparations.

[0170] Each composition described above may further contain other active components unless formulation with the antibody causes any adverse interaction.

(7) DNA transgenic animal

[0171] The present invention provides a non-human mammal bearing DNA encoding the polypeptide of the present invention, which is exogenous (hereinafter abbreviated as the exogenous DNA of the present invention) or its variant DNA (sometimes merely referred to as the exogenous variant DNA of the present invention).

[0172] Thus, the present invention provides:

(1) a non-human mammal bearing the exogenous DNA of the present invention or its variant DNA;
(2) the mammal according to (1), wherein the non-human mammal is a rodent;
(3) the mammal according to (2), wherein the rodent is mouse or rat; and,
(4) a recombinant vector bearing the exogenous DNA of the present invention or its variant DNA and capable of expressing in a mammal.

[0173] The non-human mammal bearing the exogenous DNA of the present invention or its variant DNA (hereinafter simply referred to as the DNA transgenic animal of the present invention) can be prepared by transfecting a desired DNA into an unfertilized egg, a fertilized egg, a spermatozoon, a germinal cell containing a primordial germinal cell thereof, or the like, preferably in the embryogenic stage in the development of a non-human mammal (more preferably in the single cell or fertilized cell stage and generally before the 8-cell phase), by standard means, such as the calcium phosphate method, the electric pulse method, the lipofection method, the agglutination method, the microinjection method, the particle gun method, the DEAE-dextran method etc. Also, it is possible to transfect the exogenous DNA of the present invention into a somatic cell, a living organ, a tissue cell, or the like by the DNA transfection methods,

and utilize the transformant for cell culture, tissue culture, etc. In addition, these cells may be fused with the above-described germinal cell by a publicly known cell fusion method to prepare the transgenic animal of the present invention.

[0174] Examples of the non-human mammal that can be used include bovine, swine, sheep, goats, rabbits, dogs, cats, guinea pigs, hamsters, mice, rats, and the like. Above all, preferred are rodents, especially mice (e.g., C57B1/6 strain, DBA2 strain, etc. for a pure line and for a cross line, B6C3F$_1$ strain, BDF$_1$ strain B6D2F$_1$ strain, BALB/c strain, ICR strain, etc.) or rats (Wistar, SD, etc.), since they are relatively short in ontogeny and life cycle from a standpoint of preparing model animals for human disease.

[0175] "Mammals" in a recombinant vector that can be expressed in the mammals include the aforesaid non-human mammals and human.

[0176] The exogenous DNA of the present invention refers to the DNA of the present invention that is once isolated and extracted from mammals, not the DNA of the present invention inherently possessed by the non-human mammals.

[0177] The mutant DNA of the present invention includes mutants resulting from variation (e.g., mutation, etc.) in the base sequence of the original DNA of the present invention, specifically DNAs resulting from base addition, deletion, substitution with other bases, etc. and further including abnormal DNA.

[0178] The abnormal DNA is intended to mean the DNA that expresses a polypeptide and exemplified by the DNA capable of expressing a polypeptide that suppresses the function of the normal polypeptide of the present invention.

[0179] The exogenous DNA of the present invention may be any one of those derived from a mammal of the same species as, or a different species from, the mammal as the target animal. In transfecting the DNA of the present invention, it is generally advantageous to use the DNA as a DNA construct in which the DNA is ligated downstream a promoter capable of expressing the DNA in the target animal. For example, in the case of transfecting the human DNA of the present invention, a DNA transgenic mammal that expresses the DNA of the present invention to a high level, can be prepared by microinjecting a DNA construct (e.g., vector, etc.) ligated with the human DNA of the present invention into a fertilized egg of the target non-human mammal downstream various promoters which are capable of expressing the DNA derived from various mammals (e.g., rabbits, dogs, cats, guinea pigs, hamsters, rats, mice, etc.) bearing the DNA of the present invention highly homologous to the human DNA.

[0180] As expression vectors for the polypeptide of the present invention, there are *Escherichia* coli-derived plasmids, *Bacillus subtilis*-derived plasmids, yeast-derived plasmids, bacteriophages such as λ phage, retroviruses such as Moloney leukemia virus, etc., and animal viruses such as vaccinia virus, baculovirus, etc. Of these vectors, *Escherichia coli*-derived plasmids, *Bacillus subtilis*-derived plasmids, or yeast-derived plasmids, etc. are preferably used.

[0181] Examples of these promoters for regulating the DNA expression include (1) promoters for DNA derived from viruses (e.g., simian virus, cytomegalovirus, Moloney leukemia virus, JC virus, breast cancer virus, poliovirus, etc.), and (2) promoters derived from various mammals (human, rabbits, dogs, cats, guinea pigs, hamsters, rats, mice, etc.), for example, promoters of albumin, insulin II, uroplakin II, elastase, erythropoietin, endothelin, muscular creatine kinase, glial fibrillary acidic protein, glutathione S-transferase, platelet-derived growth factor β, keratins K1, K10 and K14, collagen types I and II, cyclic AMP-dependent protein kinase βI subunit, dystrophin, tartarate-resistant alkaline phosphatase, atrial natriuretic factor, endothelial receptor tyrosine kinase (generally abbreviated as Tie2), sodium-potassium adenosine triphosphorylase (Na, K-ATPase), neurofilament light chain, metallothioneins I and IIA, metalloproteinase I tissue inhibitor, MHC class I antigen (H-2L), H-ras, renin, dopamine β-hydroxylase, thyroid peroxidase (TPO), polypeptide chain elongation factor 1α (EF-1α), β actin, α and β myosin heavy chains, myosin light chains 1 and 2, myelin base protein, thyroglobulins, Thy-1, immunoglobulins, H-chain variable region (VNP), serum amyloid component P, myoglobin, troponin C, smooth muscle α actin, preproencephalin A, vasopressin, etc. Among them, cytomegalovirus promoters, human polypeptide elongation factor 1α (EF-1α) promoters, human and chicken β actin promoters etc., which protein can highly express in the whole body, are preferred.

[0182] It is preferred that the vectors described above have a sequence for terminating the transcription of the desired messenger RNA in the DNA transgenic animal (generally termed a terminator); for example, a sequence of each DNA derived from viruses and various mammals. SV40 terminator of the simian virus, etc. are preferably used.

[0183] In addition, for the purpose of increasing the expression of the desired exogenous DNA to a higher level, the splicing signal and enhancer region of each DNA, a portion of the intron of an eukaryotic DNA may also be ligated at the 5' upstream of the promoter region, or between the promoter region and the translational region, or at the 3' downstream of the translational region, depending upon purposes.

[0184] The translational region can be prepared by a conventional DNA engineering technique in which the DNA is ligated downstream the aforesaid promoter and if desired, upstream the translation termination site, as a DNA construct capable of being expressed in the transgenic animal.

[0185] The exogenous DNA of the present invention is transfected at the fertilized egg cell stage in a manner such that the DNA is certainly present in all the germinal cells and somatic cells of the target mammal. The fact that the exogenous DNA of the present invention is present in the germinal cells of the animal prepared by DNA transfection means that all offspring of the animal prepared will maintain the exogenous DNA of the present invention in all of the germinal cells and somatic cells thereof. The offspring of the animal that inherits the exogenous DNA of the present

invention also have the exogenous DNA in all of the germinal cells and somatic cells thereof.

[0186] The non-human mammal in which the normal exogenous DNA of the present invention has been transfected can be passaged as the DNA-bearing animal under ordinary rearing environment, by confirming that the exogenous DNA is stably retained by mating.

[0187] By the transfection of the exogenous DNA of the present invention at the fertilized egg cell stage, the DNA is retained to be excess in all of the germinal and somatic cells. The fact that the exogenous DNA of the present invention is excessively present in the germinal cells of the prepared animal after transfection means that the exogenous DNA of the present invention is excessively present in all of the germinal cells and somatic cells thereof. The offspring of the animal that inherits the exogenous DNA of the present invention have excessively the exogenous DNA of the present invention in all of the germinal cells and somatic cells thereof.

[0188] By obtaining a homozygotic animal having the transfected DNA in both of homologous chromosomes and mating a male and female of the animal, all offspring can be passaged to retain the DNA.

[0189] In a non-human mammal bearing the normal DNA of the present invention, the normal DNA of the present invention has expressed to a high level, and may eventually develop hyperfunction in the function of the polypeptide of the present invention by promoting the function of endogenous normal DNA. Therefore, the animal can be utilized as a pathologic model animal for such a disease. Specifically, using the normal DNA transgenic animal of the present invention, it is possible to elucidate the mechanism of hyperfunction in the function of the polypeptide of the present invention and the pathological mechanism of the disease associated with the polypeptide of the present invention and to determine how to treat these diseases.

[0190] Furthermore, since a mammal transfected with the exogenous normal DNA of the present invention exhibits an increasing symptom of the polypeptide of the present invention librated, the animal is usable for screening of treatment agent for the disease associated with the polypeptide of the present invention.

[0191] On the other hand, non-human mammal having the exogenous abnormal DNA of the present invention can be passaged under normal breeding conditions as the DNA-bearing animal by confirming stable retention of the exogenous DNA via crossing. Furthermore, the exogenous DNA of interest can be utilized as a starting material by inserting the DNA into the plasmid described above. The DNA construct with a promoter can be prepared by conventional DNA engineering techniques. The transfection of the abnormal DNA of the present invention at the fertilized egg cell stage is preserved to be present in all of the germinal and somatic cells of the mammals to be subjected. The fact that the abnormal DNA of the present invention is present in the germinal cells of the animal after DNA transfection means that all of the offspring of the prepared animal have the abnormal DNA of the present invention in all of the germinal and somatic cells. Such an offspring that passaged the exogenous DNA of the present invention contains the abnormal DNA of the present invention in all of the germinal and somatic cells. A homozygous animal having the introduced DNA on both of homologous chromosomes can be acquired, and then by mating these male and female animals, all the offspring can be bled to have the DNA.

[0192] Since non-human mammal having the abnormal DNA of the present invention may express the abnormal DNA of the present invention at a high level, the animal may eventually be the function inactivation type inadaptability of the polypeptide of the present invention by inhibiting the function of the endogenous normal DNA and can be utilized as its disease model animal. For example, using animal transfected with the abnormal DNA of the present invention, it is possible to elucidate the mechanism of inadaptability of the polypeptide and to perform studies on a method for treatment of this disease.

[0193] More specifically, the transgenic animal of the present invention expressing the abnormal DNA of the present invention at a high level is also expected to serve as an experimental model for the elucidation of the mechanism of the functional inhibition (dominant negative effect) of a normal polypeptide by the abnormal polypeptide of the present invention in the function inactive type inadaptability of the polypeptide of the present invention.

[0194] A mammal bearing the abnormal exogenous DNA of the present invention is also expected to serve for screening a candidate drug for the treatment of the function inactive type inadaptability of the polypeptide of the present invention, since a free form of the polypeptide of the present invention is increased in such an animal.

[0195] Other potential applications of two kinds of the transgenic animals described above include:

(1) use as a cell source for tissue culture;

(2) elucidation of the relation to a polypeptide that is specifically expressed or activated by the polypeptide of the present invention, through direct analysis of the DNA or RNA in tissue of the DNA transgenic animal of the present invention or through analysis of the polypeptide tissue expressed by the DNA;

(3) research on the function of cells derived from tissues that are cultured usually only with difficulty, using cells of tissue bearing the DNA cultured by a standard tissue culture technique;

(4) screening of a drug that enhances cell functions using the cells described in (3) above; and,

(5) isolation and purification of the variant polypeptide of the present invention and preparation of an antibody thereto.

[0196] Furthermore, clinical conditions of a disease associated wit the polypeptide of the present invention, including the function inactive type inadaptability of the polypeptide of the present invention can be determined using the DNA transgenic animal of the present invention. Also, pathological findings on each organ in a disease model associated with the polypeptide of the present invention can be obtained in more detail, leading to the development of a new method for treatment as well as the research and therapy of any secondary diseases associated with the disease.

[0197] It is also possible to obtain a free DNA-transfected cell by removing each organ from the DNA transgenic animal of the present invention, mincing the organ and degrading with a polypeptide (protein) degrading enzyme such as trypsin, etc., followed by establishing the line of culturing or cultured cells. Furthermore, the DNA transgenic animal of the present invention can serve as identification of cells capable of producing the polypeptide of the present invention, and as studies on relevance to apoptosis, differentiation or propagation, or on the mechanism of signal transduction in these properties to inspect any abnormality therein. Thus, the DNA transgenic animal of the present invention can provide an effective research material for the polypeptide of the present invention and for elucidating its function and effect.

[0198] To develop a therapeutic drug for the treatment of diseases associated with the polypeptide of the present invention, including the function inactive type inadaptability of the polypeptide of the present invention, using the DNA transgenic animal of the present invention, an effective and rapid method for screening can be provided by using the method for inspection and the method for quantification, etc. described above. It is also possible to investigate and develop a method for DNA therapy for the treatment of diseases associated with the polypeptide of the present invention, using the DNA transgenic animal of the present invention or a vector capable of expressing the exogenous DNA of the present invention.

(8) Knockout animal

[0199] The present invention provides a non-human mammal embryonic stem cell bearing the DNA of the present invention inactivated and a non-human mammal deficient in expressing the DNA of the present invention.

[0200] Thus, the present invention provides:

(1) a non-human embryonic stem cell in which the DNA of the present invention is inactivated;
(2) the embryonic stem cell according to (1), wherein the DNA is inactivated by introducing a reporter gene (e.g., β-galactosidase gene derived from *Escherichia coli*) ;
(3) the embryonic stem cell according to (1), which is resistant to neomycin;
(4) the embryonic stem cell according to (1), wherein the non-human mammal is a rodent;
(5) the embryonic stem cell according to (4), wherein the rodent is mouse;
(6) a non-human mammal deficient in expressing the DNA, wherein the DNA of the present invention is inactivated;
(7) the non-human mammal according to (6), wherein the DNA is inactivated by inserting a reporter gene (e.g., β-galactosidase derived from *Escherichia coli*) therein and the reporter gene is capable of being expressed under control of a promoter for the DNA of the present invention;
(8) the non-human mammal according to (6), wherein the non-human mammal is a rodent;
(9) the non-human mammal according to claim 8, wherein the rodent is mouse; and,
(10) a method for screening a compound or its salt that promotes or inhibits the activity of a promoter to the DNA of the present invention, which comprises administering a test compound to the mammal of (7) and detecting expression of the reporter gene.

[0201] The non-human mammal embryonic stem cell in which the DNA of the present invention is inactivated refers to a non-human mammal embryonic stem cell that suppresses the ability of the non-human mammal to express the DNA by artificially mutating the DNA of the present invention, or the DNA has no substantial ability to express the polypeptide of the present invention (hereinafter sometimes referred to as the knockout DNA of the present invention) by substantially inactivating the activities of the polypeptide of the present invention encoded by the DNA (hereinafter merely referred to as ES cell).

[0202] As the non-human mammal, the same examples as described above apply.

[0203] Techniques for artificially mutating the DNA of the present invention include deletion of a part or all of the DNA sequence and insertion of or substitution with other DNA, by genetic engineering. By these variations, the knockout DNA of the present invention may be prepared, for example, by shifting the reading frame of a codon or by disrupting the function of a promoter or exon.

[0204] Specifically, the non-human mammal embryonic stem cell in which the DNA of the present invention is inactivated (hereinafter merely referred to as the ES cell with the DNA of the present invention inactivated or the knockout ES cell of the present invention) can be obtained by, for example, isolating the DNA of the present invention that the desired non-human mammal possesses, inserting a DNA fragment having a DNA sequence constructed by inserting

a drug resistant gene such as a neomycin resistant gene or a hygromycin resistant gene, or a reporter gene such as lacZ (β-galactosidase gene) or cat (chloramphenicol acetyltransferase gene), etc. into its exon site thereby to disable the functions of exon, or integrating to a chromosome of the subject animal by, e.g., homologous recombination, a DNA sequence which terminates gene transcription (e.g., polyA additional signal, etc.) in the intron between exons to, thus inhibit the synthesis of complete messenger RNA and eventually disrupt the gene (hereinafter simply referred to as targeting vector). The thus-obtained ES cells are subjected to the Southern hybridization analysis with a DNA sequence on or near the DNA of the present invention as a probe, or to PCR analysis with a DNA sequence on the targeting vector and another DNA sequence near the DNA of the present invention, which is not included in the targeting vector as primers, thereby to select the knockout ES cell of the present invention.

[0205] The parent ES cells to inactivate the DNA of the present invention by homologous recombination, etc. may be of a strain already established as described above, or may be originally established in accordance with a modification of the known method by Evans and Kaufman *supra*. For example, in the case of mouse ES cells, currently it is common practice to use ES cells of the 129 strain. However, since their immunological background is obscure, the C57BL/6 mouse or the BDF1 mouse (F1 hybrid between C57BL/6 and DBA/2), wherein the low ovum availability per C57BL/6 in the C57BL/6 mouse has been improved by crossing with DBA/2, may be preferably used, instead of obtaining a pure line of ES cells with the clear immunological genetic background and for other purposes. The BDF1 mouse is advantageous in that, when a pathologic model mouse is generated using ES cells obtained therefrom, the genetic background can be changed to that of the C57BL/6 mouse by back-crossing with the C57BL/6 mouse, since its background is of the C57BL/6 mouse, as well as being advantageous in that ovum availability per animal is high and ova are robust.

[0206] In establishing ES cells, blastocytes at 3.5 days after fertilization are commonly used. In the present invention, embryos are preferably collected at the 8-cell stage, after culturing until the blastocyte stage, the embryos are used to efficiently obtain a large number of early stage embryos.

[0207] Although the ES cells used may be of either sex, male ES cells are generally more convenient for generation of a germ cell line chimera and are therefore preferred. It is also desirable that sexes be identified as soon as possible to save painstaking culture time.

[0208] Methods for sex identification of the ES cell include the method in which a gene in the sex-determining region on the Y-chromosome is amplified by the PCR process and detected. When this method is used, one colony of ES cells (about 50 cells) is sufficient for sex-determination analysis, which karyotype analysis, for example G-banding method, requires about 10^6 cells; therefore, the first selection of ES cells at the early stage of culture can be based on sex identification, and male cells can be selected early, which saves a significant amount of time at the early stage of culture.

[0209] Second selection can be achieved by, for example, number of chromosome confirmation by the G-banding method. It is usually desirable that the chromosome number of the obtained ES cells be 100% of the normal number. However, when it is difficult to obtain the cells having the normal number of chromosomes due to physical operation etc. in cell establishment, it is desirable that the ES cell be again cloned to a normal cell (e.g., in mouse cells having the number of chromosomes being 2n = 40) after the gene of the ES cells is rendered knockout.

[0210] Although the embryonic stem cell line thus obtained shows a very high growth potential, it must be subcultured with great care, since it tends to lose its ontogenic capability. For example, the embryonic stem cell line is cultured at about 37°C in a carbon dioxide incubator (preferably about 5% carbon dioxide and about 95% air, or about 5% oxygen, about 5% carbon dioxide and about 90% air) in the presence of LIF (1-10000 U/ml) on appropriate feeder cells such as STO fibroblasts, treated with a trypsin/EDTA solution (normally approximately 0.001-0.5% trypsin/approximately 0.1-5 mM EDTA, preferably about 0.1% trypsin/about 1 mM EDTA) at the time of passage to obtain separate single cells, which are then seeded on freshly prepared feeder cells. This passage is normally conducted every 1 to 3 days; it is desirable that cells be observed at passage and cells found to be morphologically abnormal in culture, if any, be abandoned.

[0211] Where ES cells are allowed to reach a high density in mono-layers or to form cell aggregates in suspension under appropriate conditions, they will spontaneously differentiate to various cell types, for example, pariental and visceral muscles, cardiac muscle or the like [M. J. Evans and M. H. Kaufman, Nature, 292, 154, 1981; G. R. Martin, Proc. Natl. Acad. Sci. U.S.A., 78, 7634, 1981; T. C. Doetschman et al., Journal of Embryology Experimental Morphology, 87, 27, 1985]. The cells deficient in expression of the DNA of the present invention, which are obtainable from the differentiated ES cells of the present invention are useful for studying the function of the polypeptide of the present invention cytologically or molecular biologically.

[0212] The non-human mammal deficient in expression of the DNA of the present invention can be distinguished from a normal animal by measuring the mRNA amount in the subject animal by a publicly known method, and indirectly comparing the degree of expression.

[0213] As the non-human mammal, the same examples supra apply.

[0214] With respect to the non-human mammal deficient in expression of the DNA of the present invention, the DNA

of the present invention can be made knockout by transfecting a targeting vector, prepared as described above, to mouse embryonic stem cells or mouse oocytes, and conducting homologous recombination in which a targeting vector DNA sequence, wherein the DNA of the present invention is inactivated by the transfection, is replaced with the DNA of the present invention on a chromosome of a mouse embryonic stem cell or mouse embryo.

[0215] The knockout cells with the DNA of the present invention disrupted can be identified by the Southern hybridization analysis with a DNA fragment on or near the DNA of the present invention as a probe, or by PCR analysis using as primers a DNA sequence on the targeting vector and another DNA sequence of the mouse-derived DNA of the present invention, which is not included in the targeting vector. When non-human mammalian embryonic stem cells are used, a cell line wherein the DNA of the present invention is inactivated by homologous recombination is cloned; the resulting cloned cell line is injected to, e.g., a non-human mammalian embryo or blastocyst, at an appropriate stage such as the 8-cell stage. The resulting chimeric embryos are transplanted to the uterus of the pseudopregnant non-human mammal. The resulting animal is a chimeric animal composed of both cells having the normal locus of the DNA of the present invention and those having an artificially mutated locus of the DNA of the present invention.

[0216] When some germ cells of the chimeric animal have a mutated locus of the DNA of the present invention, an individual, which entire tissue is composed of cells having a mutated locus of the DNA of the present invention can be selected from a series of offspring obtained by crossing between such a chimeric animal and a normal animal, e.g., by coat color identification, etc. The individuals thus obtained are normally deficient in heterozygous expression of the polypeptide of the present invention. The individuals deficient in homozygous expression of the polypeptide of the present invention can be obtained from offspring of the intercross between the heterozygotes.

[0217] When an oocyte or egg cell is used, a DNA solution may be injected, e.g., to the prenucleus by microinjection thereby to obtain a transgenic non-human mammal having a targeting vector introduced in a chromosome thereof. From such transgenic non-human mammals, those having a mutation at the locus of the DNA of the present invention can be obtained by selection based on homologous recombination.

[0218] As described above, individuals in which the DNA of the present invention is rendered knockout permit passage rearing under ordinary rearing conditions, after the individuals obtained by their crossing have proven to have been knockout.

[0219] Furthermore, the genital system may be obtained and maintained by conventional methods. That is, by crossing male and female animals each having the inactivated DNA, homozygote animals having the inactivated DNA in both loci can be obtained. The homozygotes thus obtained may be reared so that one normal animal and two or more homozygotes are produced from a mother animal to efficiently obtain such homozygotes. By crossing male and female heterozygotes, homozygotes and heterozygotes having the inactivated DNA are proliferated and passaged.

[0220] The non-human mammal embryonic stem cell in which the DNA of the present invention is inactivated is very useful for preparing a non-human mammal deficient in expression of the DNA of the present invention.

[0221] Since the non-human mammal in which the DNA of the present invention is deficiently expressed lacks various biological activities induced by the polypeptide of the present invention, such an animal can be a disease model suspected of inactivated biological activities of the polypeptide of the present invention and thus, offers an effective study to investigate causes for and therapy for these diseases.

(8a) Method for screening of compounds that are effective for the treatment/prevention of diseases caused by deficiency, damages, etc. of the DNA of the present invention.

[0222] The non-human mammal deficient in expression of the DNA of the present invention can be employed for screening of compounds that are effective for the treatment/prevention of diseases caused by deficiency, damages, etc. of the DNA of the present invention.

[0223] That is, the present invention provides a method for screening of a compound having therapeutic/prophylactic effects for diseases caused by deficiency, damages, etc. of the DNA of the present invention, which comprises administering a test compound to the non-human mammal deficient in expression of the DNA of the present invention and observing/measuring a change occurred in the animal.

[0224] As the non-human mammal deficient in expression of the DNA of the present invention which can be employed for the screening method, the same examples as given hereinabove apply.

[0225] Examples of the test compounds include peptides, proteins, non-peptide compounds, synthetic compounds, fermentation products, cell extracts, vegetable extracts, animal tissue extracts, blood plasma and the like and these compounds may be novel compounds or publicly known compounds.

[0226] Specifically, the non-human mammal deficient in expression of the DNA of the present invention is treated with a test compound, comparison is made with an intact animal for control and a change in each organ, tissue, disease conditions, etc. of the animal is used as an index to assess the therapeutic/prophylactic effects of the test compound.

[0227] For treating a test animal with a test compound, for example, oral administration, intravenous injection, etc. are available, and the treatment is appropriately selected depending upon conditions of the test animal, properties of

the test compound, etc. Furthermore, an amount of administration for a test compound can be appropriately chosen depending on the administration route, nature of the test compound and the like.

[0228] In the case of screening a compound effective for the treatment/prevention of, e.g., tumor angiogenesis, a cancer cell is transplanted to the non-human mammal deficient in expression of the DNA of the present invention, a test compound is administered before or after the cancer cell transplantation and, a tumor marker value, tumor mass size, etc. of the animal is measured with passage of time.

[0229] The compound obtainable using the above screening method is a compound selected from the test compounds described above and exhibits a therapeutic and prophylactic effect for the diseases caused by deficiencies, damages, etc. of the polypeptide of the present invention (bone and joint diseases (e.g., arthritis deformans, chronic articular rheumatism, marble stone disease, etc.), pathological angiogenesis (e.g., tumor angiogenesis), etc.). Therefore, the compound can be employed as a safe and low toxic pharmaceutical such as an agent for the treatment and prevention of these diseases. Furthermore, compounds derived from such a compound obtainable by the screening *supra* can be employed as well.

[0230] The compound obtained by the screening above may be used in the form of salts with physiologically acceptable acids (e.g., inorganic acids or organic acids) or bases (e.g., alkali metal salts), preferably in the form of physiologically acceptable acid addition salts. Examples of such salts are salts with inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid), salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

[0231] A pharmaceutical comprising the compound obtained by the above screening method or salts thereof may be manufactured in a manner similar to the method for preparing the pharmaceutical comprising the polypeptide of the present invention described hereinabove.

[0232] Since the pharmaceutical thus obtained is safe and low toxic, it can be administered to human and another mammal (e.g., rat, mouse, guinea pig, rabbit, sheep, swine, bovine, horse, cat, dog, monkey, etc.).

[0233] The dose of the compound or its salt to be administered varies depending upon particular disease, subject to be administered, route of administration, etc., but when it is orally administered to an adult (as 60 kg body weight) for the treatment of bone and joint disease, the compound is administered generally in a dose of about 0.1 mg/day to about 100 mg/day, preferably about 1.0 mg/day to about 50 mg/day, more preferably about 1.0 mg/day to about 20 mg/day. When it is parenterally administered to an adult (as 60 kg body weight) for the treatment of bone and joint disease, the single dose of the compound varies depending upon subject to be administered, particular disease, etc., and it is advantageous to administer the composition in the form of an injectable preparation in a dose of about 0.01 mg/day to about 30 mg/day, preferably about 0.1 mg/day to about 20 mg/day, more preferably about 0.1 mg/day to about 10 mg/day. As for other animals, the composition can be administered in the above dose with converting it into that for the body weight of 60 kg.

(8b) Method for screening a compound or its salt that promotes or inhibits the activities of a promoter to the DNA of the present invention

[0234] The present invention provides a method for screening a compound or its salt that promotes or inhibits the activities of a promoter to the DNA of the present invention, which comprises administering a test compound to a non-human mammal deficient in expression of the DNA of the present invention and detecting expression of the reporter gene.

[0235] In the screening method *supra*, the non-human mammal deficient in expression of the DNA of the present invention is selected from the aforesaid non-human mammal deficient in expression of the DNA of the present invention, as an animal in which the DNA of the present invention is inactivated by introducing a reporter gene and the reporter gene is expressed under control of a promoter to the DNA of the present invention.

[0236] The same examples of the test compound described above apply to specific test compounds.

[0237] As the reporter gene, the same specific examples apply to those for this method. Preferably employed are β-galactosidase (lacZ), soluble alkaline phosphatase gene, luciferase gene and the like.

[0238] Since a reporter gene is present under control of a promoter to the DNA of the present invention in the non-human mammal deficient in expression of the DNA of the present invention wherein the DNA of the present invention is substituted with the reporter gene, the activity of the promoter can be detected by tracing expression of a substance encoded by the reporter gene.

[0239] When a part of the DNA region encoding the polypeptide of the present invention is substituted with, e.g., β-galactosidase gene (lacZ) derived from Escherichia coli, β-galactosidase is expressed in a tissue where the polypeptide of the present invention should originally be expressed, instead of the polypeptide of the present invention. Thus, the state of expression of the polypeptide can be readily observed in vivo of an animal by staining with a reagent, e.g., 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) which is a substrate for β-galactosidase. Specifically, a

mouse deficient in the polypeptide of the present invention, or its tissue section is fixed with glutaraldehyde, etc. After washing with phosphate buffered saline (PBS), the system is reacted with a staining solution containing X-gal at room temperature or about 37°C for approximately 30 minutes to an hour. After the β-galactosidase reaction is terminated by washing the tissue preparation with 1 mM EDTA/PBS solution, the color formed is observed. Alternatively, mRNA encoding lacZ may be detected in a conventional manner.

[0240] The compound or its salt obtainable by the screening method supra are compounds that are selected from the test compounds described above and that accelerate or inhibit the activity of a promoter to the DNA of the present invention.

[0241] The compound obtained by the screening method above may take the form of salts with physiologically acceptable acids (e.g., inorganic acids or organic acids) or bases (e.g., alkali metal salts), preferably in the form of physiologically acceptable acid addition salts. Examples of such salts are salts with inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid), salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and the like.

[0242] Since the compound or its salt that promotes or inhibits the activity of a promoter to the DNA of the present invention can accelerate the expression of the polypeptide of the present invention or can accelerate the function of the polypeptide of the present invention, they are useful as safe and low toxic pharmaceuticals for the treatment/prevention of diseases, for example, bone and joint diseases (e.g., arthritis deformans, chronic articular rheumatism, marble stone disease, etc.), pathological angiogenesis (e.g., tumor angiogenesis), etc.

[0243] In addition, compounds derived from the compounds obtained by the screening supra can be used as well.

[0244] The pharmaceutical composition comprising the compound or its salt obtained by the screening method can be manufactured as in the pharmaceutical comprising the polypeptide of the present invention or its salt, described above.

[0245] The thus obtained pharmaceutical is safe and low toxic, and can thus be administered to, e.g., human or mammal (e.g., rat, mouse, guinea pig, rabbit, sheep, swine, bovine, horse, cat, dog, monkey, etc.).

[0246] The dose of the compound or its salt varies depending on target disease, subject to be administered, route for administration, etc.; for example, in oral administration of the compound that promotes the activity of a promoter to the DNA of the present invention for the treatment of, e.g., bone and joint diseases, the dose is normally about 0.1 to about 100 mg, preferably about 1.0 to about 50 mg, more preferably about 1.0 to about 20 mg per day for adult (as 60 kg body weight). In parenteral administration, the single dose varies depending on subject to be administered, target disease, etc. but it is advantageous to administer, for example, the compound that accelerates the activity of a promoter to the DNA of the present invention intravenously at a daily dose of about 0.01 to about 30 mg, preferably about 0.1 to about 20 mg, more preferably about 0.1 to about 10 mg for adult (as 60 kg body weight). For other animal species, the corresponding dose as converted per 60 kg weight can be administered.

[0247] On the other hand, when the compound that inhibits the activity of a promoter to the DNA of the present invention is orally administered, the dose is normally from about 0.1 to about 100 mg, preferably about 1.0 to about 50 mg, more preferably about 1.0 to about 20 mg per day for adult (as 60 kg body weight). In parenteral administration, the single dose varies depending on subject to be administered, target disease, etc. When the compound that inhibits the activity of a promoter to the DNA of the present invention is administered to an adult (as 60 kg body weight) generally in the form of injection, it is advantageous to administer the compound intravenously at a daily dose of about 0.01 to about 30 mg, preferably about 0.1 to about 20 mg, more preferably about 0.1 to about 10 mg. For other animal species, the corresponding dose as converted per 60 kg weight can be administered.

[0248] As stated above, the non-human mammal deficient in expression of the DNA of the present invention is extremely useful for screening the compound or its salt that promotes or inhibits the activity of a promoter to the DNA of the present invention and can greatly contribute to the elucidation of causes for various diseases suspected of deficiency in expression of the DNA of the present invention and for the development of pharmaceuticals for the prevention/treatment of these diseases.

[0249] In addition, extremely large quantities of the gene encoding the polypeptide of the present invention are expressed in mouse or human, especially in the cartilage tissue. Thus, the promoter sequence of the gene is advantageously used as a promoter to express a protein of interest (an optional useful gene product, etc.) in the cartilage tissue of a non-human warm-blooded animal. Specific examples of the warm blooded animal are the same as those given above.

[0250] That is, the present invention provides a method of expressing an protein of interest (an optional useful gene product, etc.) predominantly in the cartilage tissue of a non-human warm blooded animal, which comprises ligating the protein of interest (an optional useful gene product, etc.) to the gene encoding the polypeptide comprising the amino acid sequence represented by SEQ ID NO:6, SEQ ID NO:12 or SEQ ID NO:47 at the downstream of its promoter region (3' terminal side) and inserting the protein into the non-human animal.

[0251] Examples of the protein of interest (an optional useful gene product, etc.) include useful gene products such

as cytokines (e.g., interleukin, interferon, chemokine, hematopoietic factors), growth factors (e.g., EGF (epidermal growth factor) or substances having an activity that is substantially the same as EGF (e.g., EGF, heregulin (HER2 ligand), etc.), insulin or substances having an activity that is substantially the same as insulin (e.g., insulin, IGF (insulin-like growth factor)-1, IGF-2, etc.), FGF (fibroblast growth factor) or substances having an activity that is substantially the same as FGF (e.g., aFGF, bFGF, KGF (Keratindcyte Growth Factor), HGF (Hepatocyte Growth Factor), FGF-10, etc.), other cell growth factors (e.g., CSF (colony stimulating factor), EPO (erythropoietin), IL-2 (interleukin-2), NGF (nerve growth factor), PDGF (platelet-derived growth factor), TGFβ (transforming growth factor β)), etc.), hormones (e.g., lutenizing hormone-releasing hormone (LH-RH), growth hormone, growth hormone-releasing hormone (GH-RH), prolactin, melanocyte stimulating hormone, thyroid hormone-releasing hormone, thyroid-stimulating hormone, lutenizing hormone, corpus luteum hormone, follicle-stimulating hormone, gastrin, motilin, somatostatin, secretin, glucagon, PACAP, VIP, etc., digestive enzymes (e.g., amylase, pepsinogen, lipase, etc.), antibodies to pathogen (e.g., antibodies to pathogenic bacteria such as pathogenic Salmonella, etc., antibodies to pathogenic viruses such as influenza, etc., antibodies to parasites such as Echinococcus, etc., or the like), antibacterial polypeptides (e.g., cecropin, histatin, indolicidin, protegrin, defensin, lysozyme, etc.) and the like.

[0252] In the proteins of interest described above,

(1) the cytokines are cartilage-specifically expressed, whereby, e.g., the immune activity of a non-human warm blooded animal can be potentiated or controlled; and,
(2) the growth factors are cartilage-specifically expressed, whereby; e.g., the cartilage tissue of a non-human warm blooded animal can be protected, etc.

[0253] Hereinafter, the method of expressing an protein of interest (an optional useful gene product, etc.) predominantly in the cartilage tissue of a non-human warm blooded animal, which comprises ligating DNA or RNA encoding the protein of interest (an optional useful gene product, etc.) at the downstream of promoter region of the gene encoding the polypeptide comprising the amino acid sequence represented by SEQ ID NO:6, SEQ ID NO:12 or SEQ ID NO:47 (3' terminal side) and introducing it into the non-human animal, is described below more specifically.

[0254] First, the promoter of the gene encoding the polypeptide characterized by comprising the amino acid sequence represented by SEQ ID NO:6, SEQ ID NO:12 or SEQ ID NO:47 can be obtained by publicly known methods such as colony hybridization, plaque hybridization, PCR, etc. (e.g., methods described in Molecular Cloning, 2nd (J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989), etc.). The region having the promoter activity can be identified by publicly known methods such as reporter assay, etc. (e.g., methods described in Analytical Biochemistry, vol. 188, page 245 (1990), etc.).

[0255] Next, in order to ligate a protein of interest (an optional useful gene product, etc.) at the downstream (3' terminal side) of the promoter obtained by the methods described above, the ligation can be performed by publicly known methods for constructing plasmids using T4 DNA ligase (e.g., methods described in Molecular Cloning, 2nd (J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989), etc.).

[0256] For transfecting the promoter ligated with the DNA encoding the protein of interest (an optional useful gene product, etc.) at the downstream (3' terminal side) of the promoter, there are a method using electroporation, a method using gene gun, a method using a retroviral vector (a method described in, e.g., Blood Cells, 17, 407 (1991), etc.), a method using an adenoviral vector (a method described in, e.g., Pathology, 30, 335 (1998), etc.) or the like.

[0257] When bases, amino acids, etc. are shown by abbreviations in the specification and drawings, they are represented by the codes in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature or by the common codes in the art, examples of which are shown below. For amino acids that may have the optical isomer, L form is presented unless otherwise indicated.

DNA :	deoxyribonucleic acid
cDNA :	complementary deoxyribonucleic acid
A :	adenine
T :	thymine
G :	guanine
RNA :	ribonucleic acid
mRNA :	messenger ribonucleic acid
dATP :	deoxyadenosine triphosphate
dTTP :	deoxythymidine triphosphate
dGTP :	deoxyguanosine triphosphate
dCTP :	deoxycytidine triphosphate
ATP :	adenosine triphosphate
EDTA :	ethylenediaminetetraacetic acid

SDS : sodium dodecyl sulfate
Gly : glycine
Ala : alanine
Val : valine
Leu : leucine
Ile : isoleucine
Ser : serine
Thr : threonine
Cys : cysteine
Met : methionine
Glu : glutamic acid
Asp : aspartic acid
Lys : lysine
Arg : arginine
His : histidine
Phe : phenylalanine
Tyr : tyrosine
Trp : tryptophan
Pro : proline
Asn : asparagine
Gln : glutamine
pGlu : pyroglutamic acid

[0258] The substituents, protecting groups and reagents which are frequently used in the present specification are represented by the following symbols.

Me : methyl group
Et : ethyl group
Bu : butyl group
Ph : phenyl group
Tc : thiazolidine-4(R)-carboxamide group
Tos : p-toluenesulfonyl
CHO : formyl
Bzl : benzyl
Cl_2-Bzl : 2,6-dichlorobenzyl
Bom : benzyloxymethyl
Z : benzyloxycarbonyl
Cl-Z : 2-chlorobenzyloxycarbonyl
Br-Z : 2-bromobenzyloxycarbonyl
Boc : t-butoxycarbonyl
DNP : dinitrophenol
Trt : trityl
Bum : t-butoxymethyl
Fmoc : N-9-fluorenylmethoxycarbonyl
HOBt : 1-hydroxybenztriazole
HOOBt : 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
HONB : 1-hydroxy-5-norbornene-2,3-dicarboximide
DCC : N, N'-dichlorohexylcarbodiimide

[0259] The sequence identification numbers in the sequence listing of the specification indicates the following sequence, respectively.

[SEQ ID NO:1]

[0260] This shows the base sequence of the antisense strand primer used in EXAMPLE 1.

[SEQ ID NO:2]

[0261] This shows the base sequence of the sense strand primer used in EXAMPLE 1.

[SEQ ID NO:4]

[0262] This shows the base sequence of DNA encoding human MLP precursor bearing the amino acid sequence shown by SEQ ID NO:6.

[SEQ ID NO:5]

[0263] This shows the amino acid sequence of signal sequence contained in human MLP precursor bearing the amino acid sequence shown by SEQ ID NO:6.

[SEQ ID NO:6]

[0264] This shows the amino acid sequence of human MLP precursor.

[SEQ ID NO:7]

[0265] This shows the base sequence of the antisense strand primer used in EXAMPLE 2.

[SEQ ID NO:8]

[0266] This shows the base sequence of the sense strand primer used in EXAMPLE 2.

[SEQ ID NO:10]

[0267] This shows the base sequence of DNA encoding mouse MLP precursor bearing the amino acid sequence shown by SEQ ID NO:12.

[SEQ ID NO:11]

[0268] This shows the amino acid sequence of signal sequence contained in mouse MLP precursor bearing the amino acid sequence shown by SEQ ID NO:12.

[SEQ ID NO:12]

[0269] This shows the amino acid sequence of mouse MLP precursor.

[SEQ ID NO:13]

[0270] This shows the base sequence of G3PDH-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:14]

[0271] This shows the base sequence of G3PDH-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:15]

[0272] This shows the base sequence of aggrecan-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:16]

[0273] This shows the base sequence of aggrecan-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:17]

[0274] This shows the base sequence of type II collagen-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:18]

[0275] This shows the base sequence of type II collagen-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:19]

[0276] This shows the base sequence of type X collagen-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:20]

[0277] This shows the base sequence of type X collagen-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:21]

[0278] This shows the base sequence of mouse MLP-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:22]

[0279] This shows the base sequence of mouse MLP-specific oligo DNA used in EXAMPLE 3.

[SEQ ID NO:23]

[0280] This shows the base sequence of DNA encoding human MLP bearing the amino acid sequence shown by SEQ ID NO:24.

[SEQ ID NO:24]

[0281] This shows the amino acid sequence of human MLP.

[SEQ ID NO:25]

[0282] This shows the base sequence of DNA encoding mouse MLP bearing the amino acid sequence shown by SEQ ID NO:26.

[SEQ ID NO:26]

[0283] This shows the amino acid sequence of mouse MLP.

[SEQ ID NO:27]

[0284] This shows the base sequence of the primer employed in EXAMPLES 4 and 6.

[SEQ ID NO:28]

[0285] This shows the base sequence of the primer employed in EXAMPLE 4.

[SEQ ID NO:29]

[0286] This shows the base sequence of the cDNA fragment obtained in EXAMPLE 1.

[SEQ ID NO:30]

[0287] This shows the base sequence of the cDNA fragment obtained in EXAMPLE 2.

[SEQ ID NO:31]

[0288] This shows the amino acid sequence of the synthetic peptide employed in EXAMPLE 6.

[SEQ ID NO:32]

[0289] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 6.

[SEQ ID NO:33]

[0290] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 6.

[SEQ ID NO:34]

[0291] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 6.

[SEQ ID NO:35]

[0292] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 6.

[SEQ ID NO:36]

[0293] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 6.

[SEQ ID NO:37]

[0294] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 6.

[SEQ ID NO:38]

[0295] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 6.

[SEQ ID NO:39]

[0296] This shows the partial amino acid sequence of rat MLP precursor encoded by the DNA obtained in EXAMPLE 9.

[SEQ ID NO:40]

[0297] This shows the base sequence of the DNA encoding a part of rat MLP precursor obtained in EXAMPLE 9.

[SEQ ID NO:41]

[0298] This shows the base sequence of the DNA containing the DNA encoding a part of rat MLP precursor obtained in EXAMPLE 9.

[SEQ ID NO:42]

[0299] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 9.

[SEQ ID NO:43]

[0300] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 9.

[SEQ ID NO:44]

[0301] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 9.

[SEQ ID NO:45]

[0302] This shows the base sequence of oligo DNA used as the PCR primer in EXAMPLE 9.

[SEQ ID NO:46]

[0303] This shows the base sequence of the DNA encoding rat MLP precursor having the amino acid sequence represented by SEQ ID NO:47.

[SEQ ID NO:47]

[0304] This shows the amino acid sequence of rat MLP precursor.

[SEQ ID NO:48]

[0305] This shows the base sequence of the DNA encoding rat MLP having the amino acid sequence represented by SEQ ID NO:49.

[SEQ ID NO:49]

[0306] This shows the amino acid sequence of rat MLP.

[SEQ ID NO:50]

[0307] This shows the amino acid sequence of signal sequence contained in rat MLP precursor bearing the amino acid sequence shown by SEQ ID NO:47.

[0308] *Escherichia coli* transformant XL10-Gold/pDRL128vH obtained in EXAMPLE 1 later described has been deposited with the Ministry of International Trade and Industry, Agency of Industrial Science and Technology, National Institute of Bioscience and Human-Technology (NIBH), 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan, under the Accession Number FERM BP-6750 since June 25, 1999, and with Institute for Fermentation, Osaka (IFO), 17-85, Juso-honmachi 2-chome Yodogawa-ku, Osaka, Japan, as the Accession Number IFO 16292 since June 25, 1999.

[0309] *Escherichia coli* transformant XL10-Gold/pDRL128vM obtained in EXAMPLE 2 later described has been deposited with the Ministry of International Trade and Industry, Agency of Industrial Science and Technology, National Institute of Bioscience and Human-Technology (NIBH), under the Accession Number FERM BP-6747 since June 9, 1999, and with Institute for Fermentation (IFO), as the Accession Number IFO 16293 since June 25, 1999.

[0310] *Escherichia coli* transformant XL10-Gold/pDRL128vR obtained in EXAMPLE 9 later described has been deposited with the Ministry of International Trade and Industry, Agency of Industrial Science and Technology, National Institute of Bioscience and Human-Technology (NIBH), under the Accession Number FERM BP-7167 since May 19, 2000, and with Institute for Fermentation (IFO) as the Accession Number IFO 16439 since May 26, 2000.

[0311] Hereinafter, the present invention is described in detail with reference to EXAMPLES, but not intended to limit the scope of the present invention thereto. The gene manipulation procedures using *Escherichia coli* were performed according to the methods described in the Molecular Cloning.

EXAMPLE 1

Cloning of cDNA encoding human MLP precursor protein

[0312] Cloning of cDNA encoding human MLP precursor protein was carried out by performing 5' RACE (Rapid Amplification of cDNA End) and 3' RACE using human fetal brain-derived poly(A)$^+$ RNA, according to the following procedures. From 1 μg of human fetal brain-derived poly(A)$^+$ RNA (Clonetech Inc.), 1st strand cDNA was synthesized using anchored primer having poly(T) following the restriction enzyme sites and Superscript II MMLV reverse transcriptase (Gibco BRL Inc.). Then, the anchored primer was added to the 1st strand cDNA at the 3' end using RNA ligase (Takara Shuzo Co., Ltd.). Next, 5' RACE was carried out using as an antisense strand primer the oligo DNA shown by SEQ ID NO:1, and 3' RACE was carried out using-as a sense strand primer the oligo DNA shown by SEQ ID NO:2, whereby the 5' upstream sequence and the 3' downstream sequence starting from the respective primers were obtained, respectively. Base sequencing of each of the double stranded DNAs obtained indicates the presence of overlapping common sequences, which reveals that the two sequences are derived from the same gene. Therefore, the respective cDNA fragments obtained by 5' RACE and 3' RACE were ligated with the common sequence segment

to finally obtain poly(A)$^+$-containing cDNA fragment of 923 base pairs (bp) in the full-length shown by SEQ ID NO:29. This cDNA fragment encoded a novel human MLP precursor protein of 128 amino acids represented by SEQ ID NO: 6, containing a typical signal sequence of 18 amino acid residues shown by SEQ ID NO:5. This human MLP precursor protein had the highest homology to human MIA precursor protein, and the positions of four cysteine residues coincided (FIG. 1) but the homology between the two was only 23.4% on an amino acid level.

[0313] Plasmid pDRL128vH bearing the DNA encoding human MLP precursor protein obtained this EXAMPLE was transfected to Escherichia coli XL10-Gold to acquire transformant Escherichia coli XL10-Gold/pDRL128vH.

EXAMPLE 2

Cloning of cDNA encoding mouse MLP precursor protein

[0314] Cloning of cDNA encoding mouse MLP precursor protein was carried out by performing 5' RACE (Rapid Amplification of cDNA End) and 3' RACE, using poly(A)$^+$ RNA derived from the mouse fetus of 17.5 days old, as in the cloning of cDNA encoding human MLP precursor protein. After fractionation of the total RNA from the mouse fetus of 17.5 days old by the guanidine thiocyanate method, the total RNA was applied to oligo(dT) span column (Pharmacia) to prepare poly(A)$^+$ RNA. From 1 μg of poly(A)$^+$ RNA derived from the mouse fetus of 17.5 days old, 1st strand cDNA was synthesized using anchored primer having poly(T) following the restriction enzyme sites and Superscript II MMLV reverse transcriptase (Gibco BRL Inc.). Then, the anchored primer was added to the synthesized 1st strand cDNA at the 3' end, using RNA ligase (Takara Shuzo Co., Ltd.). The sequences of the primers used for 5' RACE and 3' RACE were prepared based on the sequence of AA222797, which was found in the public EST (Expressed Sequence Tag) by treating as a query the base sequence of cDNA encoding human MLP precursor protein obtained in EXAMPLE 1 and which was the unique EST considered to contain the 3' region of cDNA encoding mouse MLP precursor protein. 5' RACE was carried out using as an antisense strand primer the oligo DNA shown by SEQ ID NO:7, and 3' RACE was carried out using as a sense strand primer the oligo DNA shown by SEQ ID NO:8, whereby the 5' upstream sequence and the 3' downstream sequence starting from the respective primers were obtained, respectively. Base sequencing of each of the double stranded DNAs obtained indicates the presence of overlapping common sequences, which reveals that the two sequences are derived from the same gene. Therefore, the respective cDNA fragments obtained by 5' RACE and 3' RACE were ligated with the common sequence segment to finally obtain poly(A)$^+$ chain-containing cDNA fragment of 947 base pairs (bp) in the full-length (SEQ ID NO:30). This cDNA fragment encoded a novel mouse MLP precursor protein of 128 amino acids represented by SEQ ID NO:12, containing a typical signal sequence of 18 amino acid residues shown by SEQ ID NO:11, as in human MLP precursor protein. In this mouse MLP precursor protein, the positions of four cysteine residues, which are present in human and mouse MIA precursor proteins and human MLP precursor protein, all coincided (FIG. 1). Also, the homology of mouse MLP precursor protein to human MLP precursor protein reached 84.3% on an amino acid level, and but the homology between mouse MIA precursor protein and mouse MLP precursor protein was only 22.6%.

[0315] Plasmid pDRL128vM bearing the DNA encoding mouse MLP precursor protein obtained this EXAMPLE was transfected to Escherichia coli XL10-Gold to acquire transformant Escherichia coli XL10-Gold/pDRL128vM.

EXAMPLE 3

Expression of MLP in cartilage differentiation model in vitro

[0316] Mouse embryonic tumor-derived cell line ATDC5 has been used as an in vitro cartilage differentiation model, since ATDC5 can retain the property of precursor cartilage cells extremely well, can induce cartilage differentiation in a high rate by incubation in the presence of insulin, and can simulate all stages of cartilage differentiation observed in subsequent osteogenesis (Cell Diff. Dev., 30:109-116, 1990, J. Cell Biol., 133:457-468, 1996, J. Bone Min. Res., 10: S234, 1995). Thus, a change in expression of various genes at each stage of the differentiation was monitored by performing RT-PCR according to the procedures below. First, ATDC5 cells collected from each stage of the differentiation model incubation system were lysed in a homogeneous liquid ISOGEN (Nippon Gene Co., Ltd.) containing phenol and guanidine thiocyanate, and chloroform was added to the lysate. By centrifugation, the aqueous fraction containing RNA was acquired and isopropanol was further added thereto. The mixture was agitated, again centrifuged and precipitated to obtain the purified total RNAs. Next, using AMV Reverse Transcriptase XL and Random 9 mers in TAKARA RNA PCR Kit (AMV) Ver. 2.1 (Takara Shuzo Co., Ltd.), each of the total RNAs to be tested was reverse transcribed to obtain cDNAs. Then, using these cDNAs as a template DNA and further using as a housekeeping gene G3PDH (glyceraldehyde 3-phosphate dehydrogenase)-specific oligo DNA (SEQ ID NO:13, SEQ ID NO:14), or cartilage differentiation marker gene-specific oligo DNA (aggrecan (SEQ ID NO:15, SEQ ID NO:16), type II collagen (SEQ ID NO:17, SEQ ID NO:18), type X collagen (SEQ ID NO:19, SEQ ID NO:20)) as a primer DNA, PCR was carried out in

the reaction system of TaKaRa Ex Taq™ (Takara Shuzo Co., Ltd.). The resulting reaction products were separated by agarose gel electrophoresis and the amounts produced were compared. As the result, the expression patterns shown in TABLE 1 corresponding to the respective differentiation stages of cartilage cells could be detected. Now, using as a primer DNA oligo DNA specific to cDNA encoding mouse MLP precursor protein (SEQ ID NO:21, SEQ ID NO:22) and otherwise under the same reaction conditions, RT-PCR was carried out in the cDNA sample group. The amount of mouse MLP precursor mRNA was markedly increased from stage 2 to stage 4. This reveals that the gene of MLP precursor is of such a nature that its expression increases at the initial stages of cartilage differentiation.

TABLE 1

STAGE	1	2	3	4	5	6	7
MLP	+	+++	+++	+++	++	++	+
Aggrecan	+	++	++	++	+++	++	++
TypeII collagen	+	++	+++	+++	+++	++	++
TypeX collagen	+	+	++	++	+++	+++	+++
G3PDH	++	++	++	++	++	++	++

(The number of symbol + in the table denotes differences in the amount of each gene expressed in each stage of differentiation; the larger the number, the more the amount expressed.)

EXAMPLE 4

Expression of mouse MLP-FLAG fused protein in COS7 cells and its detection

[0317] In order to verify that MLP is a secretory protein, mouse MLP was examined by the following procedures through expression of mouse MLP-FLAG fused protein in COS7 cells and its detection. First, two primer DNAS were chemically synthesized based on the base sequence of cDNA encoding mouse MLP precursor polypeptide obtained in EXAMPLE 2. One is shown by 5'-CGAATTCCCACCATGGCAAGGATATTGATTCTTTTGCTTG-3' (SEQ ID NO:27) and is oligo DNA containing the sense sequence of +1 to +28 (wherein the translation initiation site is made +1) bearing the anchored sequence containing the restriction enzyme EcoRI recognition site at the 5' end. Another is shown by 5' - GTACAGTCGACTTCACAGAAGAAGTCAATATCCGTGGTTG-3' (SEQ ID NO:28) and is oligo DNA bearing the anchored sequence containing the restriction enzyme SalI recognition site with the antisense sequence of +355 to +378 at the 3' end. Using as a template plasmid pDRL128vM obtained in EXAMPLE 2 and further using these two primer DNAs and TaKaRa LA Taq™ (Takara Shuzo Co., Ltd.), amplification was performed with a thermal cycler GeneAmp™ PCR system 9700 (Perkin-Elmer Inc.), which included first allowing to stand at 98°C for 30 seconds followed by repeating 25 cycles set to include 98°C for 10 seconds, 55°C for 20 seconds and 72°C for 2 minutes as one cycle. Finally extension was performed at 72°C for 5 minutes. The DNA fragment thus obtained was purified, truncated with restriction enzymes EcoRI and SalI, and then purified again. The purified product was inserted into and ligated with the EcoRI and SalI sites of expression vector pCAN618FLAG for animal cells. pCAN618FLAG derived from plasmid vector pCAN618 and having a neomycin resistant gene as a selection marker can express a protein of interest under control of very early gene enhancer of cytomegalovirus and β-actin promoter downstream the enhancer, by inserting the DNA fragment encoding the protein of interest into its cloning sites, i.e., the EcoRI and SalI sites. Moreover, pCAN618FLAG is also capable of expressing the protein of interest as a FLAG fused protein, by adjusting its reading frame to the base sequence encoding FLAG epitope sequence of 8 amino acids (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) located immediately after the SalI site and termination codon. The PCR cloning DNA fragment described above was inserted into pCAN618FLAG for the purpose of expressing the fused protein of the full length mouse MLP precursor and FLAG epitope (one Val residue is inserted therebetween). Thus, expression vector plasmid pMMLP-F was obtained.

[0318] Next, 1.2 x 10^5 COS7 cells were charged in a 6-well plate and incubated for 24 hours in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and the expression plasmid pMMLP-F (0.4 μg per well) described above was transfected to the cells using Lipofectamine (Gibco BRL). Twenty four hours after the transfection, the medium was replaced by a fresh DMEM medium and then further 5 hours later by FBS-free Opti-MEM (Gibco BRL). After incubation for 36 hours, the culture supernatant and the cell extract were obtained. The cells in the cell extract were washed twice with phosphate buffered saline (PBS), lysed and extracted with Tris SDS sample-buffer solution. On the other hand, the culture supernatant was appropriately concentrated by ultrafiltration (molecular weight 3000 cut off) and the concentrate was mixed with an equal volume of Tris SDS sample buffer solution.

These samples were heat-treated and then electrophoresed on 15%-25% SDS-polyacrylamide gel. The protein was again transferred from the gel onto a PVDF membrane (Amersham Pharmacia Biotech Inc.). Next, the PVDF membrane was blocked for an hour with Block Ace (Snow Brand Milk Products Co., Ltd.) followed by reacting for 2 hours with anti-FLAG monoclonal antibody (10 μg/ml; Kodak) in PBS containing 0.05% Tween 20 (PBS-T). After washing three times with PBS-T, the reaction product was reacted in PBS-T for an hour with horseradish peroxidase-labeled anti-mouse IgG goat antibody (Amersham Pharmacia Biotech Inc., 5000-fold dilution). After washing 5 times with PBS-T, chemical light emission was detected using ECLplus color forming kit (Amersham Pharmacia Biotech Inc.) and ECL film (Amersham Pharmacia Biotech Inc.). As the result, the gene product of about 14 kDa was detected both in the cell extract and in the culture supernatant, clearly showing that mouse MLP-FLAG fused protein was expressed and secreted in COS7 cells.

[0319] Next, the N-terminal amino acid sequence of the mouse MLP-FLAG fused protein was sequenced. First, affinity chromatography was performed using Anti-FLAG™ M2-Agarose Affinity Gel (Sigma Co.) to collect the acidic eluate fraction (eluted with Glycine-HCl buffer (pH 3.5)) from the culture supernatant of COS7 cells containing mouse MLP-FLAG expressed by a modification of the above method. After the fraction was concentrated, the concentrate was electrophoresed on SDS-polyacrylamide gel as described before in this EXAMPLE, followed by CBB staining. Only a single band corresponding to the protein of interest, i.e., mouse MLP-FLAG was observed. The concentrate sample of the same fraction was subjected to electrophoresis in a similar manner. The protein was transferred from the gel onto PVDF membrane and then applied to a pulse liquid amino acid sequencer Procise CLC491 (PE Biosystems Inc.) to determine the N-terminal amino acid sequence. As the result, the amino acid residues of 1. histidine, 2. glycine, 3. valine and 4. phenylalanine were detected in this order, respectively, from the left hand, to coincide with the N-terminal sequence of mouse MLP represented by SEQ ID NO:26. The foregoing results reveal that in mouse MLP-FLAG protein, the signal sequence of N-terminal 18 amino acid residues in mouse MLP-FLAG precursor protein was cleaved and secreted from COS7 cells into medium as mouse MLP-FLAG mature protein starting with the 19th histidine residue.

EXAMPLE 5

Establishment of mouse MLP-FLAG fused protein-expressing CHL-K1 cell line

[0320] Mouse MLP-FLAG fused protein-expressing CHL-K1 cell line was established by the following procedures. On a plastic Petri dish of 10 cm in diameter, 3.3 x 10^4 CHOI-K1 cells were incubated for 24 hours in F-12 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), and the expression plasmid pMMLP-F (1.5 μg per well) obtained in EXAMPLE 4 was transfected to the cells by the calcium phosphate method (CellPhect Transfection Kit (Amersham Pharmacia Biotech Inc.). Twelve hours after the transfection, the cells were washed twice with FBS-free F-12 medium and subjected to glycerol shock for 3 minutes using 3 ml of isotonic HEPES solution (pH 7.5) containing 15% glycerol. The cells were again washed twice with FBS-free F-12 medium and incubated in F-12 medium supplemented with FBS for further 12 hours. The medium was then replaced by F-12 selection medium supplemented with 500 mg/L Geneticin (Gibco BRL) and 10% FBS (hereinafter selection medium). Ten days after, Geneticin-resistant colonies formed in the Petri dish were transferred to a 24-well plate, respectively, followed by incubation in selection medium for 3 days. Next, the cells proliferated in the selection medium were transferred to a 6-well plate and incubated in selection medium for further 4 days. The medium was replaced by 1 ml of Opti-MEM (Gibco BRL) supplemented with 0.02% CHAPS and 0.1 mM p-ABSF (Wako Pure Chemical Industries Co., Ltd.). After incubation for further 48 hours, the culture supernatant was recovered. The resulting supernatant was concentrated through Centricon YM-3 ultrafiltration membrane (Amicon Inc.), and the concentrate was mixed with an equal volume of Tris SDS sample buffer solution. The sample was heat-treated at 95°C for 5 minutes and then electrophoresed on 18% SDS-polyacrylamide gel. The electrophoresed protein was further transferred from the gel onto a nylon membrane. Next, the nylon membrane was blocked for an hour with Block Ace (Snow Brand Milk Products Co., Ltd.) followed by reacting for an hour with anti-FLAG antibody (1/2000 dilution, SIGMA) in PBS containing 0.05% Tween 20 (PBS-T). After washing 5 times with PBS-T, the reaction product was reacted in PBS-T for an hour with HRP-labeled anti-mouse IgG sheep antibody (1/2000 dilution, Amersham Pharmacia Biotech Inc.). After washing 5 times with PBS-T, chemical light emission was detected using ECL color forming kit (Amersham Pharmacia Biotech Inc.) and Hyperfilm ECL (Amersham Pharmacia Biotech Inc.). As the result, the objective gene product of about 16 kDa was detected in the largest quantity in the culture supernatant of the cells derived from CHO-K1/mMLP.FLAG#2-1 strain. Therefore, the cell line was selected as mouse MLP-FLAG fused protein-expressing CHO-K1 cell line.

EXAMPLE 6

Preparation of MLP antiserum and detection of recombinant MLP protein using the antiserum

[0321] The anti-MLP antiserum was prepared by the following procedures. First, a synthetic peptide (Val-Lys-Glu-Gln-Arg-Val-Tyr-Gln-Glu-Ala-Thr-Lys-Glu-Ile-Pro-Thr-Thr-Asp-Ile-Asp-Cys) represented by SEQ ID NO:31, which is a peptide chain corresponding to the amino acid sequence from the 105th valine to the 124th aspartic acid in mouse MLP precursor protein further added with one cysteine at the C terminus of the protein, was chemically synthesized by a publicly known method. The synthetic peptide was coupled to KLH (keyhole limpet hemocyanin) as a carrier, and it was injected to rabbit for immunization. After immunization was repeated 7 times in total, whole blood was collected and fractionated by a publicly known method to obtain the serum fraction. Sodium azide as a preservative (final concentration of 0.1%) was added to the serum fraction, which was used as anti-MLP antiserum.

[0322] Next, the reactivity of the antiserum to various recombinant proteins was examined by Western blot analysis. First, in addition to the mouse MLP-FLAG fused protein described in EXAMPLE 4, the respective expression vector plasmids, i.e., mouse MLP protein (no FLAG tag), human MLP-FLAG fused protein, human MLP protein (no FLAG tag), mouse MIA-FLAG fused protein and mouse MIA protein (no FLAG tag), were constructed. These plasmids were constructed as in EXAMPLE 4, by inserting the previously PCR-cloned DNA fragment of interest into the EcoRI and SalI sites which are the cloning sites of pCAN618FLAG. The respective base sequences of the primer DNA set used for the respective PCR reactions are the base sequences represented, respectively, by SEQ ID NO:27 and SEQ ID NO:32 for mouse MLP protein (no FLAG tag), by SEQ ID NO:33 and SEQ ID NO:34 for human MLP-FLAG fused protein, by SEQ ID NO:33 and SEQ ID NO:35 for human MLP protein (no FLAG tag), by SEQ ID NO:36 and SEQ ID NO:37 for mouse MIA-FLAG fused protein and by SEQ ID NO:36 and SEQ ID NO:38 for mouse MIA protein (no FLAG tag). As a template DNA, there was used, respectively, pDRL128vM for mouse MLP protein (no FLAG tag) as in EXAMPLE 4, pDRL128vH obtained in EXAMPLE 1 for human MLP-FLAG fused protein and human MLP protein (no FLAG tag), and cDNA prepared from mouse melanoma cell line B16 for mouse MIA-FLAG fused protein and mouse MIA protein (no FLAG tag). Transfection of the respective expression vector plasmids thus obtained to COS-7 cells and the Western blot analysis on the respective culture supernatants using anti-FLAG antibody were performed in a manner similar to the procedures of EXAMPLE 4. The Western blot analysis using the anti-MLP antiserum was performed in a similar manner to the procedures of the Western blot analysis using the anti-FLAG antibody, except that the antiserum (1000-fold dilution) was used as a primary antibody and as a secondary antibody, horseradish peroxidase labeled anti-rabbit IgG antibody (Amersham Pharmacia Biotech; 5000-fold dilution) was used.

[0323] FIG.2 shows the results of the Western blot analysis using the anti-FLAG antibody and FIG. 3 shows the results of the Western blot analysis using the anti-MLP antibody. The anti-MLP antiserum showed cross reactivity with both mouse MLP and human MLP, which are antigen peptide-derived proteins, and the reactivity was almost the same. Also, mouse MLP-FLAG fused protein and human MLP-FLAG fused protein were reacted with the antiserum, indicating that it was not affected even in the presence of FLAG tag. On the other hand, no signal was detected at all with mouse MIA-FLAG fused protein or mouse MIA protein (no FLAG tag), and the results reveal that the antiserum was specific to the MLP molecular species.

[0324] Furthermore, the mouse MLP-FLAG expressing CHO cell line acquired in EXAMPLE 5 using the antiserum was subjected to immune staining by a modification of publicly known methods. The results are shown in FIG. 4. In the control experiment using rabbit serum prior to immunization, each cell was not stained but all cells were stained when the antiserum was used. This reveals that the antiserum was also reactive with denatured MLP protein.

EXAMPLE 7

Expression of MLP protein in cartilage tissue

[0325] Using the anti-MLP antiserum obtained in EXAMPLE 6, it was attempted to detect MLP protein in the cartilage tissue. As a test specimen, mouse (BALB/c) femoral head cartilage was used after freeze-destructing with liquid nitrogen, extracting with TRIS SDS β ME SAMPLE BUFFER (Daiichi Kagaku Yakuhin K.K.) and removing the residue by centrifugation. A sample in an amount corresponding to that derived from one mouse per lane was electrophoresed on SDS-PAGE (15-25%) followed by Western blot analysis using anti-MLP antiserum as in EXAMPLE 6. The results reveal that since a signal was detected at almost the same electrophoretic position-as in mouse MLP recombinant protein, MLP protein was expressed in the cartilage tissue.

[0326] In order to examine expression of human MLP mRNA in various human tissues other than the cartilage tissue, a probe was prepared by the method of Multiprime DNA labeling system (Amersham Pharmacia Biotech: RPN. 1601Y) using the DNA fragment encoding the human MLP precursor protein obtained in EXAMPLE 1 and [α-^{32}P] dCTP (Amersham Pharmacia Biotech: 6000 Ci/mmol), and hybridization with Human MTE™ Array (CLONTECH Inc.: #7775-1)

was performed using the probe (specific activity of 1.3 x 10^{10} cpm/μg). The hybridization was carried out under the conditions according to the manual attached to the array membrane. The final washing was conducted with 0.1 x SSC and 0.1% SDS at 55°C and detection was made using BAS-2000 (Fuji Photo Film Co., Ltd.). As the result, signals observed on the array were human chromosomal DNA for control (100 ng, 500 ng) and only trace spots of nigra and fetal brain, but any of the signals was around the detection limit. It was thus judged that the amount of expression was extremely low at the transcription stage. Therefore, it was strongly suggested that MLP protein would be specifically expressed in the cartilage tissue.

EXAMPLE 8

Effect of adding MLP recombinant protein on change in expression of various genes in the cartilage differentiation model in vitro

[0327] In the cartilage differentiation model in vitro using the ATDC5 cells described in EXAMPLE 3, the effect of adding MLP recombinant protein on change in expression of various genes was examined. The MLP recombinant protein obtained as in EXAMPLE 4 by performing affinity chromatography according to a publicly known method using anti-FLAG antibody and purifying/concentrating from the culture supernatant of mouse MLP-FLAG fused protein-expressed COS-7 cells, was used as a specimen. The protein was added to ATDC5 cells every two other days from the first day when the model system was set, followed by incubation for 10 days. The cells were then recovered, and expression of each gene was examined by RT-PCR as in EXAMPLE 3. As the result, suppressed expression of each marker gene showing the increased amount of expression with differentiation, such as aggrecan, type II collagen, type X collagen, etc. was noted in the protein-added cell group. On the other hand, any significant affect was not noted with change in expression of PTH/PTHr receptor that acts suppressively on differentiation of cartilage. This result reveals that MLP protein acts suppressively on cartilage differentiation in this model system using ATDC5.

EXAMPLE 9

Analysis of gene encoding rat MLP precursor protein (rMLP)

[0328] First, the DNA fragment encoding a part of rMLP was obtained by the following PCR procedures. That is, 20 μl of a solution mixture containing 4 pmol each of oligo DNA shown by SEQ ID NO:21 as a sense strand primer and oligo DNA shown by SEQ ID NO:22 as an antisense strand primer, further containing 2 μl of 10 x Advantage™ 2 PCR Buffer (Clonetech Inc.), 0.4 μl of 50 x dNTP mix (Clonetech Inc.), 0.4 μl of 50 x Advantage 2 Polymerase Mix (Clonetech Inc.) and 2 μl of a solution of SD(IGS) rat pituitary-derived cDNA as a template DNA was prepared. Using a thermal cycler (GeneAmp™ PCR sytem model 9700 (Perkin-Elmer, Inc.)), PCR was carried out according to the program which comprises treating at 95°C for a minute, repeating 35 cycles set to include 95°C for 10 seconds, then 54°C for 10 seconds and 72°C for a minute, and then performing extension at 72°C for 3 minutes. After completion of the reaction, the solution was subjected to electrophoresis using 2.0% agarose gel, and the gel was stained with SYBR™ Green I nucleic acid gel stain (Molecular Sieve Inc.). It was confirmed that a band corresponding to DNA amplified by PCR was found at the position of about 300 bp when converted on the molecular weight marker. The DNA fragment was recovered using QIAquick Gel Extraction Kit (Qiagen), and subjected to TA cloning using PCR™ 2.1-Topo (Invitrogen Inc.) to determine its base sequence. The plasmid was transfected to competent cells of Escherichia coli Epicurian Coli XL10-Gold™ strain (Stratagene Co.). A clone bearing the foreign DNA fragment-inserted plasmid was selected from colonies of ampicillin-resistant transformants appeared on an ampicillin-containing LB agar medium, and the plasmid DNA, pDRL128vR, was prepared from the clone. In order to determine the base sequence of the inserted DNA, sequencing using ABI PRISM™ BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer, Inc.) was carried out by a thermal cycler (GeneAmp™ PCR system model 9700 (Perkin-Elmer, Inc.)) under the conditions according to the brochure attached, using pDRL128vR as a template DNA and commercially available DNA (Bca BEST Primer RV-P (Takara Shuzo Co., Ltd.)) as a sequencing primer. Thereafter, the reaction sample was analyzed by a DNA sequencer ABI PRISM™ 377 (Perkin-Elmer, Inc.).

[0329] Thus, the DNA fragment of 307 base pairs shown by SEQ ID NO:41 containing the DNA fragment of 261 base pairs shown by SEQ ID NO:40 encoding a part of novel rat MLP precursor protein of 87 amino acids shown by SEQ ID NO:39 was contained in pDRL128vR.

[0330] Turning next to the gene encoding the protein, genome walking was performed to examine the structure more upstream the 5' end and more downstream the 3' end than the base sequence above. As a test material, Rat GenomeWalker™ Kit (Clonetech Inc.) was used, and the procedures were carried out according to the protocol attached to the kit, except for using TaKaRa Ex Taq™ (Takara Shuzo Co.. Ltd.) as an enzyme for PCR. First, two oligo DNAS (rMLPGWFI (SEQ ID NO:42) and rMLPGWF2 (SEQ ID NO:43)) corresponding to a part of the base sequence shown

by SEQ ID NO:40 and two oligo DNAs (rMLPGWR1 (SEQ ID NO:44) and rMLPGWR2 (SEQ ID NO:45)) complimentary to a part of the base sequence above were chemically synthesized, respectively, as gene specific primers. As the primers, rMLPGWF1 was used in the first PCR reaction for acquiring the 3' downstream DNA, and in the following nested PCR, rMLPGWF2 was employed. In the first PCR for acquiring the 5' upstream DNA, rMLPGWR1 was used and, rMLPGWR2 was used in the following nested PCR. As to the amplified DNA fragments obtained by these reactions, the respective base sequences were analyzed, while making comparison in homology to the base sequences of cDNAs encoding human and mouse MLP precursor proteins, based on the above primer sequence sites as the start. As the result, it has become clear from the identified primary structure of genome that rat MLP precursor protein is a protein composed of 128 amino acid residues represented by SEQ ID NO:47 encoded by DNA of 384 bases shown by SEQ ID NO:46. The homology of rat MLP precursor protein to human MLP precursor protein and mouse MLP precursor protein on an amino acid level reached 84.3% and 96.0%, respectively, but the homology of rat MIA precursor protein to rat MLP precursor protein was only 26.5%. Rat MLP is a protein composed of 110 amino acid residues represented by SEQ ID NO:49 encoded by DNA of 330 bases represented by SEQ ID NO:48, and it is considered that a signal peptide composed of 18 amino acids as in mouse MLP, represented by SEQ ID NO:50, would be processed and produced from rat MLP precursor protein.

[0331] Plasmid pDRL128vR bearing the DNA encoding a part of rat MLP precursor protein obtained in this EXAMPLE was transfected into Escherichia coli XL10-Gold to obtain transformant, Escherichia coli XL10-Gold/pDRL128vR.

Industrial Applicability

[0332] The polypeptide of the present invention and DNA encoding the same can be used for the diagnosis, treatment, prevention, etc. of, e.g., bone and joint diseases and pathological angiogenesis. Further, the polypeptide of the present invention is useful as a reagent for screening a compound or its salt that promotes or inhibits the activity of the polypeptide of the present invention. Furthermore, the antibody to the polypeptide of the present invention is capable of recognizing the polypeptide of the present invention specifically and can thus be used for the detection, quantification, neutralization, etc. of the polypeptide of the present invention in a test sample fluid.

[0333] Further by using the promoter of the present invention, a large quantity of a protein (an optional useful gene product, etc.) can be expressed predominantly in the cartilage of non-human warm-blooded animal and hence, can contribute to the field of gene therapy.

SEQUENCE LISTINGS

<110> Takeda Chemical Industries, Ltd.

<120> Novel Polypeptide and its Use

<130> 2622WOOP

<150>1999-06-30

<151>JP 11-186718

<160> 48

<210> 1

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 1

CGCAGAAGAA GTCAATATCC GTGGTG 26

<210> 2

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 2

CAGCGTGTGT ACCAGGAAGC TACCAA 26

<210> 4

<211> 384

<212> DNA

<213> Human

<400> 4

ATGGCAAGAA TATTGTTACT TTTCCTCCCG GGTCTTGTGG CTGTATGTGC TGTGCATGGA 60

ATATTTATGG ACCGTCTAGC TTCCAAGAAG CTCTGTGCAG ATGATGAGTG TGTCTATACT 120

ATTTCTCTGG CTAGTGCTCA AGAAGATTAT AATGCCCCGG ACTGTAGATT CATTAACGTT 180

AAAAAAGGGC AGCAGATCTA TGTGTACTCA AAGCTGGTAA AAGAAAATGG AGCTGGAGAA 240

TTTTGGGCTG GCAGTGTTTA TGGTGATGGC CAGGACGAGA TGGGAGTCGT GGGTTATTTC 300

CCCAGGAACT TGGTCAAGGA ACAGCGTGTG TACCAGGAAG CTACCAAGGA AGTTCCCACC 360

ACGGATATTG ACTTCTTCTG CGAG 384

<210> 5

<211> 18

<212> PRT

<213> Human

<400> 5

Met Ala Arg Ile Leu Leu Leu Phe Leu Pro Gly Leu Val Ala Val Cys
1 5 10 15

Ala Val

18

<210> 6

<211> 128

<212> PRT

<213> Human

<400> 6

Met Ala Arg Ile Leu Leu Leu Phe Leu Pro Gly Leu Val Ala Val Cys
1 5 10 15

Ala Val His Gly Ile Phe Met Asp Arg Leu Ala Ser Lys Lys Leu Cys
20 25 30

Ala Asp Asp Glu Cys Val Tyr Thr Ile Ser Leu Ala Ser Ala Gln Glu
35 40 45

Asp Tyr Asn Ala Pro Asp Cys Arg Phe Ile Asn Val Lys Lys Gly Gln
50 55 60

Gln Ile Tyr Val Tyr Ser Lys Leu Val Lys Glu Asn Gly Ala Gly Glu
65 70 75 80

Phe Trp Ala Gly Ser Val Tyr Gly Asp Gly Gln Asp Glu Met Gly Val
85 90 95

Val Gly Tyr Phe Pro Arg Asn Leu Val Lys Glu Gln Arg Val Tyr Gln
100 105 110

Glu Ala Thr Lys Glu Val Pro Thr Thr Asp Ile Asp Phe Phe Cys Glu
115 120 125 128

<210> 7

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 7

CACACAGCAC GTAGTCGCAG TTGG 24

<210> 8

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 8

AACTTGGTGA AGGAGCAGCG TGTA 24

<210> 10

<211> 384

<212> DNA

<213> Mouse

<400> 10

ATGGCAAGGA TATTGATTCT TTTGCTTGGG GGCCTTGTGG TTCTATGTGC CGGGCATGGT 60

GTATTTATGG ATAAACTTTC TTCTAAGAAG TTGTGTGCGG ATGAGGAGTG TGTCTATACT 120

ATTTCTCTGG CAAGAGCACA GGAAGATTAC AATGCCCCAG ACTGTAGGTT CATCGATGTC 180

AACAAAGGGC AGCAGATCTA TGTTTACTCC AAGCTGGTAA CAGAAAACGG AGCTGGAGAG 240

TTTTGGGCTG GCAGTGTTTA TGGTGACCAC CAGGATGAGA TGGGAATTGT AGGTTATTTC 300

CCCAGCAACT TGGTGAAGGA GCAGCGTGTA TACCAGGAGG CCACCAAGGA GATCCCAACC 360

ACGGATATTG ACTTCTTCTG TGAA 384

<210> 11

<211> 18

<212> PRT

<213> Mouse

<400> 11

Met Ala Arg Ile Leu Ile Leu Leu Leu Gly Gly Leu Val Val Leu Cys
1 5 10 15

Ala Gly
18

<210> 12

<211> 128

<212> PRT

<213> Mouse

<400> 12

Met Ala Arg Ile Leu Ile Leu Leu Leu Gly Gly Leu Val Val Leu Cys
1 5 10 15

Ala Gly His Gly Val Phe Met Asp Lys Leu Ser Ser Lys Lys Leu Cys
20 25 30

Ala Asp Glu Glu Cys Val Tyr Thr Ile Ser Leu Ala Arg Ala Gln Glu
35 40 45

Asp Tyr Asn Ala Pro Asp Cys Arg Phe Ile Asp Val Lys Lys Gly Gln
50 55 60

Gln Ile Tyr Val Tyr Ser Lys Leu Val Thr Glu Asn Gly Ala Gly Glu
65 70 75 80

Phe Trp Ala Gly Ser Val Tyr Gly Asp His Gln Asp Glu Met Gly Ile
85 90 95

Val Gly Tyr Phe Pro Ser Asn Leu Val Lys Glu Gln Arg Val Tyr Gln
100 105 110

Glu Ala Thr Lys Glu Ile Pro Thr Thr Asp Ile Asp Phe Phe Cys Glu
115 120 125 128

<210> 13

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 13

ACCACAGTCC ATGCCATCAC 20

<210> 14

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 14

TCCACCACCC TGTTGCTGTA 20

<210> 15

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 15

CTACCGCGTG CGCCCATCAT CAGA 24

<210> 16

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 16

GGGAGGCCGG TTTGGTTGGG GTAGA 25

<210> 17

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 17

CACACTGGTA AGTGGGGCAA GACCG 25

<210> 18

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 18

GGATTGTGTT GTTTCAGGGT TCGGG 25

<210> 19

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 19

ACCCCCTGGC CCCTCTGGA 20

<210> 20

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 20

ATCTCACCTT TAGCCCCTGG AATG 24

<210> 21

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 21

GCCGGGCATG GTGTATTTAT 20

<210> 22

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 22

GATCTCCTTG GTGGCCTCCT GGTAT 25

<210> 23

<211> 330

<212> DNA

<213> Human

<400> 23

CATGGAATAT TTATGGACCG TCTAGCTTCC AAGAAGCTCT GTGCAGATGA TGAGTGTGTC 60

TATACTATTT CTCTGGCTAG TGCTCAAGAA GATTATAATG CCCCGGACTG TAGATTCATT 120

AACGTTAAAA AAGGGCAGCA GATCTATGTG TACTCAAAGC TGGTAAAAGA AAATGGAGCT 180

GGAGAATTTT GGGCTGGCAG TGTTTATGGT GATGGCCAGG ACGAGATGGG AGTCGTGGGT 240

TATTTCCCCA GGAACTTGGT CAAGGAACAG CGTGTGTACC AGGAAGCTAC CAAGGAAGTT 300

CCCACCACGG ATATTGACTT CTTCTGCGAG 330

<210> 24

<211> 110

<212> PRT

<213> Human

<400> 24

His Gly Ile Phe Met Asp Arg Leu Ala Ser Lys Lys Leu Cys Ala Asp
5 10 15

Asp Glu Cys Val Tyr Thr Ile Ser Leu Ala Ser Ala Gln Glu Asp Tyr
20 25 30

Asn Ala Pro Asp Cys Arg Phe Ile Asn Val Lys Lys Gly Gln Gln Ile
35 40 45

Tyr Val Tyr Ser Lys Leu Val Lys Glu Asn Gly Ala Gly Glu Phe Trp
50 55 60

Ala Gly Ser Val Tyr Gly Asp Gly Gln Asp Glu Met Gly Val Val Gly
65 70 75 80

Tyr Phe Pro Arg Asn Leu Val Lys Glu Gln Arg Val Tyr Gln Glu Ala
85 90 95

Thr Lys Glu Val Pro Thr Thr Asp Ile Asp Phe Phe Cys Glu
100 105 110

<210> 25

<211> 330

<212> DNA

<213> Mouse

<400> 25

CATGGTGTAT TTATGGATAA ACTTTCTTCT AAGAAGTTGT GTGCGGATGA GGAGTGTGTC 60

TATACTATTT CTCTGGCAAG AGCACAGGAA GATTACAATG CCCCAGACTG TAGGTTCATC 120

GATGTCAAGA AAGGGCAGCA GATCTATGTT TACTCCAAGC TGGTAACAGA AAACGGAGCT 180

GGAGAGTTTT GGGCTGGCAG TGTTTATGGT GACCACCAGG ATGAGATGGG AATTGTAGGT 240

TATTTCCCCA GCAACTTGGT GAAGGAGCAG CGTGTATACC AGGAGGCCAC CAAGGAGATC 300

CCAACCACGG ATATTGACTT CTTCTGTGAA 330

<210> 26

<211> 110

<212> PRT

<213> Mouse

<400> 26

His Gly Val Phe Met Asp Lys Leu Ser Ser Lys Lys Leu Cys Ala Asp
5 10 15

Glu Glu Cys Val Tyr Thr Ile Ser Leu Ala Arg Ala Gln Glu Asp Tyr
20 25 30

Asn Ala Pro Asp Cys Arg Phe Ile Asp Val Lys Lys Gly Gln Gln Ile
35 40 45

Tyr Val Tyr Ser Lys Leu Val Thr Glu Asn Gly Ala Gly Glu Phe Trp
50 55 60

Ala Gly Ser Val Tyr Gly Asp His Gln Asp Glu Met Gly Ile Val Gly
65 70 75 80

Tyr Phe Pro Ser Asn Leu Val Lys Glu Gln Arg Val Tyr Gln Glu Ala

85 90 95

Thr Lys Glu Ile Pro Thr Thr Asp Ile Asp Phe Phe Cys Glu

100 105 110

<210> 27

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 27

CGAATTCCCA CCATGGCAAG GATATTGATT CTTTTGCTTG 40

<210> 28

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 28

GTACAGTCGA CTTCACAGAA GAAGTCAATA TCCGTGGTTG 40

<210> 29

<211> 923

<212> DNA

<213> Human

<400> 29

GTCAGAGTTC AAGTTAAAAC AGAAAAAAGG AAGATGGCAA GAATATTGTT ACTTTTCCTC 60

CCGGGTCTTG TGGCTGTATG TGCTGTGCAT GGAATATTTA TGGACCGTCT AGCTTCCAAG 120

AAGCTCTGTG CAGATGATGA GTGTGTCTAT ACTATTTCTC TGGCTAGTGC TCAAGAAGAT 180

TATAATGCCC CGGACTGTAG ATTCATTAAC GTTAAAAAAG GGCAGCAGAT CTATGTGTAC 240

TCAAAGCTGG TAAAAGAAAA TGGAGCTGGA GAATTTTGGG CTGGCAGTGT TTATGGTGAT 300

GGCCAGGACG AGATGGGAGT CGTGGGTTAT TTCCCCAGGA ACTTGGTCAA GGAACAGCGT 360

GTGTACCAGG AAGCTACCAA GGAAGTTCCC ACCACGGATA TTGACTTCTT CTGCGAGTAA 420

TAAATTAGTT AAAACTGCAA ATAGAAAGAA AACACCAAAA ATAAAGAAAA GAGCAAAAGT 480

GGCCAAAAAA TGCATGTCTG TAATTTTGGA CTGACGTTTT AAGAATTTGT TACCTTACAG 540

AAGAGCAAGG GCTTAGGGGT TGGAGGTGGC AGATAAAAGA GGATTTTCAA CTCAAATCTT 600

GTTTCCTGCT GGCCTGGTCT GCCCACGAGC TAGAGCGGGG AAATGTTGAG CTCAAATGGG 660

TAAATTGAGA CCAGAAAATT ATTTTTTCAA CCTAGAGAAT CTCCTCTTAC AGGGGGATGC 720

ATATAACAGA TCATGTATGT GTAGTTATTT CTAAGTAGTA ATTCTTCCCA GCTCTTTGAT 780

TTGCCATATA TAAAATAGGT GGGTCGTATG TCTTCCCTTT AGACATGATG TTTTCTACTC 840

ATTTGTCTCT CTGGCCAATT GAATTATTAA TAAAAGGTCT GTATTATCAA AGAAAAAAAA 900

AAAAAAAAAA AAAAAAAAAA AAA 923

<210> 30

<211> 947

<212> DNA

<213> Mouse

<400> 30

AAGAAGGAAG ATGGCAAGGA TATTGATTCT TTTGCTTGGG GGCCTTGTGG TTCTATGTGC 60

CGGGCATGGT GTATTTATGG ATAAACTTTC TTCTAAGAAG TTGTGTGCGG ATGAGGAGTG 120

TGTCTATACT ATTTCTCTGG CAAGAGCACA GGAAGATTAC AATGCCCCAG ACTGTAGGTT 180

CATCGATGTC AAGAAAGGGC AGCAGATCTA TGTTTACTCC AAGCTGGTAA CAGAAAACGG 240

AGCTGGAGAG TTTTGGGCTG GCAGTGTTTA TGGTGACCAC CAGGATGAGA TGGGAATTGT 300

AGGTTATTTC CCCAGCAACT TGGTGAAGGA GCAGCGTGTA TACCAGGAGG CCACCAAGGA 360

GATCCCAACC ACGGATATTG ACTTCTTCTG TGAATAAGAA ATTAATTAAA ACAGCAGATA 420

AAACAGAAAC ACCAGTGATG AAGAAGAGAA GAAGTGGAAA TAACTGAACC TGTGTATCCG 480

TACCTTCCTG GCTTTATTTG GTGGCAGGAG GTTGGAGCTT GAAGGTGCTA AGATATGGAA 540

ATTGTCAACT CAGTCTTGTT TACTCTTGCC CCGGTCTTTC CACCAACTGC GACTAAGTGC 600

TGTGTGAATC ATATAGGTCA TTTATAACCC AATACTTAGC TTTCAGCGAG GAGAATCTTT 660

ATTTACTCAG TGATGAACAT ATAAGGTGTT TTATCTGTAG TTATTTCTAA ATGGTCATTC 720

TCCCCAGCTC TGACTCCATG TCCTTAAGCT TGCTGAGTTA GAAGTCTGAC TTTTGGGTGT 780

GTTTTCTGTT ATTTGTCTCT CTGGTCATGT GAAGTCTTAA TAATGTATTT GTCATGATAA 840

CTTCCTATTG TTACTTTTTA TATCTGATGC CCTTGGATAG AAGAATGTTA GGTATAAAAC 900

AAGTTTTTGT ACTCCCAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAA 947

<210> 31

<211> 21

<212> PRT

<213> Mouse

<400> 31

Val Lys Glu Gln Arg Val Tyr Gln Glu Ala Thr Lys Glu Ile Pro Thr
5 10 15

Thr Asp Ile Asp Cys
20

<210> 32

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 32

GTACAGTCGA CTTATTCACA GAAGAAGTCA ATATCCGTGG T 41

<210> 33

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 33

CGAATTCCCA CCATGGCAAG AATATTGTTA CTTTTCCTC 39

<210> 34

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 34

GTACAGTCGA CCTCGCAGAA GAAGTCAATA TCCGTGGT 38

<210> 35

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 35

GTACAGTCGA CTTACTCGCA GAAGAAGTCA ATATCCGTGG T 41

<210> 36

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 36

CGAATTCCCA CCATGGTGTG GTCCCCAGTG CTCCTT 36

<210> 37

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 37

GTACAGTCGA CCTGGCAGTA GAAATCCCAT TGATCGGT 38

<210> 38

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 38

GTACAGTCGA CCTGGCAGTA GAAATCCCAT TGATCGGT 38

<210> 39

<211> 87

<212> PRT

<213> Rat

<400> 39

Asp Lys Leu Ser Ser Lys Lys Leu Cys Ala Asp Glu Glu Cys Val Tyr
5 10 15

Thr Ile Ser Leu Ala Arg Ala Gln Glu Asp Tyr Asn Ala Pro Asp Cys
20 25 30

Arg Phe Ile Asn Val Lys Lys Gly Gln Gln Ile Tyr Val Tyr Ser Lys
35 40 45

Leu Val Thr Glu Asn Gly Ala Gly Ala Phe Trp Ala Gly Ser Val Tyr
50 55 60

Gly Asp His Gln Asp Glu Met Gly Ile Val Gly Tyr Phe Pro Ser Asn
65 70 75 80

Leu Val Arg Glu Gln Arg Val
85

<210> 40

<211> 261

<212> DNA

<213> Rat

<400> 40

GGATAAACTT TCTTCTAAGA AGTTGTGTGC AGATGAGGAG TGTGTCTATA CCATTTCTCT 60

GGCAAGAGCA CAGGAAGACT ACAATGCCCC GGACTGTAGG TTCATCAATG TCAAGAAAGG 120

GCAGCAGATC TATGTTTATT CCAAGCTGGT AACAGAAAAT GGAGCTGGGG CATTCTGGGC 180

TGGCAGTGTT TATGGTGACC ACCAGGATGA GATGGGAATT GTGGGTTATT TCCCCAGCAA 240

CTTGGTTAGA GAGCAACGAG T 261

<210> 41

<211> 307

<212> DNA

<213> Rat

<400> 41

GCCGGGCATG GTGTATTTAT GGATAAACTT TCTTCTAAGA AGTTGTGTGC AGATGAGGAG 60

TGTGTCTATA CCATTTCTCT GGCAAGAGCA CAGGAAGACT ACAATGCCCC GGACTGTAGG 120

TTCATCAATG TCAAGAAAGG GCAGCAGATC TATGTTTATT CCAAGCTGGT AACAGAAAAT 180

GGAGCTGGGG CATTCTGGGC TGGCAGTGTT TATGGTGACC ACCAGGATGA GATGGGAATT 240

GTGGGTTATT TCCCCAGCAA CTTGGTTAGA GAGCAACGAG TATACCAGGA GGGCCACCAA 300

GGAGATC 307

<210> 42

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 42

CACCAGGATG AGATGGGAAT TGTGGGTTAT

<210> 43

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 43

GGGTTATTTC CCCAGCAACT TGGTTAGAGA

<210> 44

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 44

AGACACACTC CTCATCTGCA CACAACTTC

<210> 45

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223>

<400> 45

CTCCTCATCT GCACACAACT TCTTAGAAGA

<210> 46

<211> 384

<212> DNA

<213> Rat

<400> 46

ATGGCAAGAA TATTGATTCT TTGCTTGGG GGCCTTGTGG CTCTCTGTGC CGGGCATGGC 60

ATGTTTATGG ATAAACTTTC TTCTAAGAAG TTGTGTGCAG ATGAGGAGTG TGTCTATACC 120

ATTTCTCTGG CAAGAGCACA GGAAGACTAC AATGCCCCGG ACTGTAGGTT CATCAATGTC 180

AAGAAAGGGC AGCAGATCTA TGTTTATTCC AAGCTGGTAA CAGAAAATGG AGCTGGGGCA 240

TTCTGGGCTG GCAGTGTTTA TGGTGACCAC CAGGATGAGA TGGGAATTGT GGGTTATTTC 300

CCCAGCAACT TGGTTAGAGA GCAACGAGTG TACCAGGAGG CCACCAAGGA GATTCCAACC 360

ACGGATATTG ACTTCTTCTG TGAA 384

<210> 47

<211> 128

<212> PRT

<213> Rat

<400> 47

Met Ala Arg Ile Leu Ile Leu Leu Leu Gly Gly Leu Val Ala Leu Cys
5 10 15

Ala Gly His Gly Met Phe Met Asp Lys Leu Ser Ser Lys Lys Leu Cys
20 25 30

Ala Asp Glu Glu Cys Val Tyr Thr Ile Ser Leu Ala Arg Ala Gln Glu
35 40 45

Asp Tyr Asn Ala Pro Asp Cys Arg Phe Ile Asn Val Lys Lys Gly Gln
50 55 60

Gln Ile Tyr Val Tyr Ser Lys Leu Val Thr Glu Asn Gly Ala Gly Ala
65 70 75 80

Phe Trp Ala Gly Ser Val Tyr Gly Asp His Gln Asp Glu Met Gly Ile
85 90 95

Val Gly Tyr Phe Pro Ser Asn Leu Val Arg Glu Gln Arg Val Tyr Gln
100 105 110

Glu Ala Thr Lys Glu Ile Pro Thr Thr Asp Ile Asp Phe Phe Cys Glu
115 120 125

<210> 48

<211> 330

<212> DNA

<213> Rat

<400> 48

CATGGCATGT TTATGGATAA ACTTTCTTCT AAGAAGTTGT GTGCAGATGA GGAGTGTGTC 60

TATACCATTT CTCTGGCAAG AGCACAGGAA GACTACAATG CCCCGGACTG TAGGTTCATC 120

AATGTCAAGA AAGGGCAGCA GATCTATGTT TATTCCAAGC TGGTAACAGA AAATGGAGCT 180

GGGGCATTCT GGGCTGGCAG TGTTTATGGT GACCACCAGG ATGAGATGGG AATTGTGGGT 240

TATTTCCCCA GCAACTTGGT TAGAGAGCAA CGAGTGTACC AGGAGGCCAC CAAGGAGATT 300

CCAACCACGG ATATTGACTT CTTCTGTGAA 330

<210> 49

<211> 110

<212> PRT

<213> Rat

<400> 49

His Gly Met Phe Met Asp Lys Leu Ser Ser Lys Lys Leu Cys Ala Asp
5 10 15

Glu Glu Cys Val Tyr Thr Ile Ser Leu Ala Arg Ala Gln Glu Asp Tyr
20 25 30

Asn Ala Pro Asp Cys Arg Phe Ile Asn Val Lys Lys Gly Gln Gln Ile
35 40 45

Tyr Val Tyr Ser Lys Leu Val Thr Glu Asn Gly Ala Gly Ala Phe Trp
50 55 60

Ala Gly Ser Val Tyr Gly Asp His Gln Asp Glu Met Gly Ile Val Gly
65 70 75 80

Tyr Phe Pro Ser Asn Leu Val Arg Glu Gln Arg Val Tyr Gln Glu Ala
85 90 95

Thr Lys Glu Ile Pro Thr Thr Asp Ile Asp Phe Phe Cys Glu
100 105 110

<210> 50

<211> 18

<212> PRT

<213> Rat

<400> 50

Met Ala Arg Ile Leu Ile Leu Leu Leu Gly Gly Leu Val Ala Leu Cys
5 10 15

Ala Gly
18

Claims

1. A polypeptide containing an amino acid sequence, which is the same or substantially the same as the amino acid sequence represented by SEQ ID NO:24, its amide or ester, or a salt thereof.

2. The polypeptide, its amide or ester, or a salt thereof, according to claim 1, which contains an amino acid sequence that is the same or substantially the same as the amino acid sequence represented by SEQ ID NO:6.

3. The polypeptide or its amide or ester, or a salt thereof, according to claim 1, wherein substantially the same amino acid sequence represented by SEQ ID NO:24 is the amino acid sequence represented by SEQ ID NO:26.

4. The polypeptide or its amide or ester, or a salt thereof, according to claim 2, wherein substantially the same amino acid sequence represented by SEQ ID NO:6 is the amino acid sequence represented by SEQ ID NO:12.

5. The polypeptide or its amide or ester, or a salt thereof, according to claim 1, wherein substantially the same amino acid sequence represented by SEQ ID NO:24 is the amino acid sequence represented by SEQ ID NO:49.

6. The polypeptide or its amide or ester, or a salt thereof, according to claim 2, wherein substantially the same amino acid sequence represented by SEQ ID NO:6 is the amino acid sequence represented by SEQ ID NO:47.

7. A DNA comprising a DNA bearing a base sequence encoding the polypeptide according to claim 1.

8. The DNA according to claim 6, wherein the base sequence encoding the polypeptide according to claim 1 is the base sequence represented by SEQ ID NO:23.

9. The DNA according to claim 6, wherein the base sequence encoding the polypeptide according to claim 1 is the base sequence represented by SEQ ID NO:4.

10. The DNA according to claim 6, wherein the base sequence encoding the polypeptide according to claim 1 is the base sequence represented by SEQ ID NO:25.

11. The DNA according to claim 6, wherein the base sequence encoding the polypeptide according to claim 1 is the base sequence represented by SEQ ID NO:10.

12. The DNA according to claim 6, wherein the base sequence encoding the polypeptide according to claim 1 is the base sequence represented by SEQ ID NO:48.

13. The DNA according to claim 6, wherein the base sequence encoding the polypeptide according to claim 1 is the base sequence represented by SEQ ID NO:46.

14. A recombinant vector comprising the DNA according to claim 6.

15. A transformant transformed with the recombinant vector according to claim 14.

16. A method for manufacturing the polypeptide or its amide or ester, or a salt thereof, according to claim 1, which comprises culturing said transformant according to claim 15 and producing the polypeptide according to claim 1.

17. An antibody to the polypeptide or its amide or ester, or a salt thereof, according to claim 1.

18. A method of screening a compound or its salt that promotes or inhibits the activity of the polypeptide or its salt according to claim 1, which comprises using the polypeptide, its amide or ester, or a salt thereof, according to claim 1.

19. A kit for screening a compound or its salt that promotes or inhibits the activity of the polypeptide, its amide or ester, or a salt thereof, according to claim 1, comprising the polypeptide or its salt according to claim 1.

20. A compound or its salt that promotes or inhibits the activity of the polypeptide, its amide or ester, or a salt thereof, according to claim 1, which is obtainable using the screening method according to claim 18 or using the screening kit according to claim 19.

21. A pharmaceutical comprising a compound or its salt that promotes or inhibits the activity of the polypeptide, its amide or ester, or a salt thereof, according to claim 1, which is obtainable using the screening method according to claim 18 or using the screening kit according to claim 19.

22. A pharmaceutical comprising the polypeptide, its amide or ester, or a salt thereof, according to claim 1.

23. An agent for the prevention/treatment of bone and joint diseases or pathologic angiogenesis, comprising the polypeptide, its amide or ester, or a salt thereof, according to claim 1.

24. A diagnostic agent comprising the antibody according to claim 17.

Fig.1

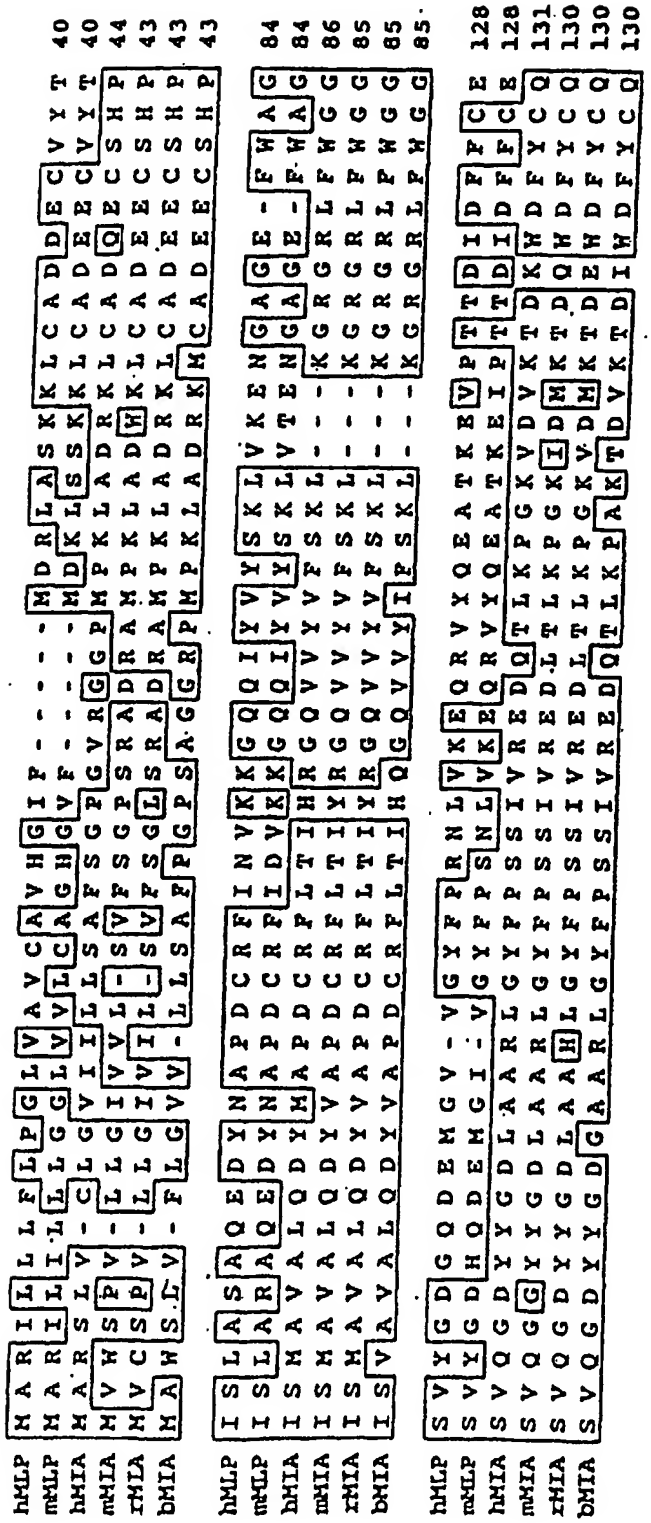

hMLP
mMLP
hMIA
mMIA
rMIA
bMIA
40
40
44
43
43
43
84
84
86
85
85
85
128
128
131
130
130
130

Fig.2

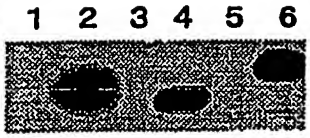


Fig.3

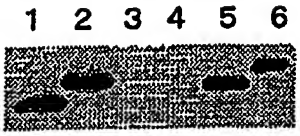
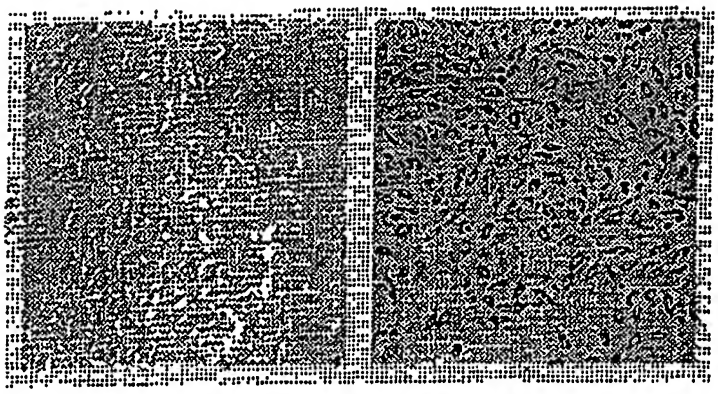


Fig.4



pre-immune anti-MLP

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/04278

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12N15/12, C12N5/10, C12P21/02, C07K14/47, C07K16/18, A61K45/00, A61K38/17, A61K39/395, A61K49/16, A61P19/02, A61P19/08, A61K31/7088//(C12P21/02, C12R1:19)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C12N15/12, C12N5/10, C12P21/02, C07K14/47, C07K16/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI(DIALOG),Genbank/EMBL/DDBJ/GeneSeq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BLESCH A.et al., "Cloning of a novel malignant melanoma-derived growth-regulatory protein,MIA." Cancer Res.(1994), Vol.54., pp.5695-5701	1-24
X	BOSSERHOFF A.-K. et al., "Structure and promoter analysis of the gene encoding the human melanoma-inhibiting protein MIA." J.Biol.Chem.(1996), Vol.271, pp.490-495	1-24
X	WO, 95/3328, A2 (BOEHRINGER MANNHEIM GMBH), 02 February, 1995 (02.02.95) & JP, 09-500531, A & DE, 4425481, A1 & EP, 710248, A1 & US, 5770366, A	1-24
P,X	WO, 99/32614, A1 (Genetics Inst. Inc.), 01 July, 1999 (01.07.99) & AU, 9919349, A & EP, 1037970, A1	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 October, 2000 (06.10.00)	17 October, 2000 (17.10.00)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

BA

OPIC
OFFICE DE LA PROPRIÉTÉ INTELLECTUELLE DU CANADA

CIPO
CANADIAN INTELLECTUAL PROPERTY OFFICE

Ottawa Hull K1A 0C9

(11) (E) **1,340,132**

(21) 617,068

(22) 1996/12/16

(45) 1998/11/17

(64) Reissue of No 1,311,413
Dated 1992/12/15

(52) 167-103.44

(51) Int.Cl.[6] A61K 38/24; A61K 38/09

(19)(CA) **REISSUED CANADIAN PATENT** (12)

(54) Composition and Method for Producing Superovulation in Cattle

(72) Donaldson, Lloyd E.
U.S.A.

(73) AUSA INTERNATIONAL, INC.
U.S.A.

(57) 86 Claims

ABSTRACT OF THE DISCLOSURE

A hormone composition is provided herein for producing superovulation in mammals, e.g., cattle, goats, swine, sheep, horses, exotic mammals or humans. The composition has a particular ratio of follicle stimulating hormone (FSH) and luteinizing hormone (LH) which produces an optimum ovulation response in mammals and promotes out of season breeding and twinning. The composition can be produced from mammal pituitary glands or by recombinant DNA procedures and can, in a preferred embodiment, be preserved in a phosphate buffered saline solution of thymol.

serum. The gonadotropic hormones have become known as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) based on their effects on ovarian follicular activity. The activity of FSH and LH preparations are usually measured by bioassay and compared to a reference standard. Typical reference standards are the National Institute of Health standards which are designated NIH-FSH-S1 and NIH-LH-S1 for FSH and LH respectively.

Treatment of cattle with gonadotropins leads to ovulation of numerous ova instead of the usual one. Gonadotropin treatment is usually initiated between days 9 and 14 of the estrus cycle (estrus is day 0), causing ovarian follicles to grow. Two or three days after the start of treatment, prostaglandin F_{2a} or an analog is injected to terminate the luteal phase of the estrus cycle prematurely by lysing the corpus luteum; about 2 days later estrus occurs. Estrus lasts about half a day, ovulation occurs about half a day after the end of estrus, and fertilization probably occurs a few hours after ovulation.

Before prostaglandins became available, superovulation was initiated about 4 to 5 days before the end of the estrus cycle, a time that could not be estimated accurately. Availability of prostaglandin F_{2a} has improved the efficacy of superovulation and has also provided flexibility in scheduling donors.

Because the best bulls are usually propagated only with frozen semen, artificial insemination is used routinely for valuable cows. Sometimes mixtures of semen from two or three bulls are used with superovulation, and the progeny are sorted out after birth on the basis of blood type.

Bovine embryos move from the oviduct to the uterus 4 to 5 days after estrus (3 to 4 days after ovulation), although in superovulated cows a few remain in the oviduct through day 7. A high percentage of embryos can usually be recovered nonsurgically from the uterus six or more days after the beginning of estrus. Recovery of embryos from the oviduct requires surgery and, therefore, is recommended only in certain cases of infertility.

To recover embryos, a Foley catheter is inserted

through the cervix into the uterus by palpating through the wall of the rectum with one hand as is done for artificial insemination. The latex catheter consists of three channels for inflow, outflow, and inflation of a balloon-like cuff that prevents the escape of fluid after insertion. Each uterine horn is filled and emptied five to ten times with 30 to 200 milliliters of fluid each time, according to the size of the uterus. The embryos are flushed out with this fluid into large graduated cylinders. Embryos can be filtered or allowed to settle for 30 minutes and can then be located under a stereomicroscope by searching through an aliquot from the bottom of the cylinder. They are then stored in small containers until transfer.

Embryos from the one-cell to the early blastocyst stage (7 to 8 days after estrus) are between 120 and 140 micrometers in diameter exclusive of the zona pellucida. Between days 8 and 10, they double in diameter, hatch from the zona pellucida, and then grow to 20 centimeters or more in length by day 18. Since bovine embryos form no intimate attachment to the uterus before day 18, they can be recovered nonsurgically until this time, although they are increasingly prone to damage after day 14. It appears that a larger number of normal embryos can be obtained nonsurgically 6 to 8 days after estrus than at other times.

It has been shown (Donaldson et al., Theriogenology 23, 189 (1985); Donaldson et al., Theriogenology 25, 749 (1986)), that luteinizing hormone (LH) contamination of follicle stimulating hormone (FSH) reduces the superovulation response in cattle. The excessive variability in superovulation response in cattle to a standardized quantity of FSH was reported in 1944 (Hammond et al., Journal Agricultural Science 34, 1 (1944)), but it was not until forty years later when the dynamics of follicular development and the response to exogenous gonadotropins was described (Monneaux et al., Theriogenology 19, 55 (1983); Moor et al., Theriogenology 21, 103 (1984)) that more reliable superovulation techniques began to be developed. It has been shown that commercial FSH preparations have high and variable LH contents (Murphy et

al., Theriogenology 21, 117 (1984); Lindsell et al., Theriogenology 25, 167 (1986)). Excess LH in a superovulatory hormone has been shown to cause premature stimulation of the oocyte (Moor et al. Theriogenology 21, 103 (1984)). Rat oocytes produced by superovulation have been shown to exhibit reduced fertilization rates (Walton et al., Journal of Reproduction and Fertility 67, 91 (1983); Walton et al., Journal of Reproduction and Fertility 67,309 (1983)). Low fertilization rates in superovulated cattle have been shown not to have resulted from the quantity of semen used or the number of times the cow was bred (Donaldson, Veterinary Record 117, 35 (1985)).

It has been shown that normal preovulatory progesterone (P4) LH and FSH concentrations are necessary for optimal embryo production from superovulated cows (Donaldson, Theriogenology 23, 441 (1985); Calleson et al., Theriogenology 25, 71 (1986)). Abnormal concentrations of P4, LH and FSH are followed by abnormal follicular/oocyte maturation and lowered embryo production.

A commonly available FSH preparation manufactured by Armour Pharmaceutical Co. and known as FSH-P is a crude pituitary extract having a high and variable LH content. The LH content has been measured and the FSH/LH ratio has been found to be less than 100. Armour Pharmaceutical Co. is the assignee of U.S. Patent Nos. 2,799,621 and 3,119,740 which relate to the preparation of FSH-P.

U.S. Patent No. 2,799,621 to Steelman is directed to a method for recovering both adrenocorticotropin (ACTH) and gonadotropins (FSH and LH) from the same batch of pituitary material.

U.S. Patent No. 3,119,740 to Steelman, et al. is directed to a method for preparing follicle stimulating hormone (FSH) free from contaminant physiological factors.

Development of reliable superovulation methods in cattle for producing adequate and predictable numbers of embryos has been slow (Moor et al., Theriogenology 21:103-116 (1984)). As noted above, the excessive variability in the numbers of ova shed in response to a standardized amount of injected hormone was first reported

in 1944 (Hammond et al., Journal Agricultural Science 34, 1 (1944)), but it was not until 1983 (Monneaux et al., Theriogenology 19, 55 (1983); Moor et al., Theriogenology 21:103-116 (1984)) that the reasons for this variability began to be understood. The dynamics of follicular development during the bovine estrus cycle, the response to exogenous gonadotropins (Moor et al., Theriogenology 19, 55 (1983); Moor et al., Theriogenology 21:103-116 (1984)), and the differences in the relative abundance of FSH and LH activity in gonadotropin preparations (Murphy et al., Theriogenology 21:117-125 (1984)) contribute to this variability. The ratio of FSH to LH activity in the various hormone preparations used for superovulation varies between batches of Armour's FSH-P and between FSH-P and pregnant mare serum gonadotropin (PMSG) (Monneaux et al., Theriogenology 19:55-64 (1983); Murphy et al., Theriogenology 22:205-212 (1984)). FSH stimulates the growth of granulosa cells in preantral and small antral follicles (Monneaux et al., Theriogenology 19:55-64 (1983)) and reverses the process of atresia in follicles over 1.7 mm in diameter (Moor et al., Theriogenology 21:103-116 (1984)). In the normal cow, the LH surge is responsible for the resumption of meiosis in the preovulatory oocyte, and the reduction in the high LH content of pituitary gonadotropin preparations should decrease premature activation of oocytes during superovulation (Moor et al., Theriogenology 21:103-116 (1984)). A previous study (Donaldson, Theriogenology 22:205-212 (1984)) showed that embryo production depended upon the dose of FSH-P. As the dose increased above an optimal 28 mg, three embryo production endpoints declined: the number of transferable embryos, the total embryos recovered, and the percent transferable. The number of collections at which no embryos were recovered also increased.

Considering the potential immunological reactions that might be encountered, employing bovine preparations in treatments involving cattle seems appropriate. The purification of bovine FSH has been reported (Beckers et al., Biochemie 59:825-831 (1977); Cheng, Biochem. J.

159:651-659 (1976); Grimek et al. Endocrinology 104:140-147 (1979)). However, the content of FSH in bovine pituitaries is relatively low and the recovery with purification is generally poor. Porcine pituitaries are as readily available and the FSH content seems more amenable to extraction and processing. Indeed, commercially available preparations of porcine origin have been widely used in veterinary medicine. Methods for the purification of porcine FSH have also been described (Closset et al., Eur. J. Biochem. 86:105-113 (1978); Whitley et al., Endocrinology 102:1874-1886 (1978)). The amino acid sequence for porcine FSH has been proposed (Closset., Eur. J. Biochem. 86:115-120 (1978), but there is no reported sequence for the bovine hormone.

The present invention is directed to a hormone composition for producing superovulation in cattle that avoids the above-mentioned disadvantages which are characteristic of the prior art.

Thus, by one broad aspect of the present invention, a composition of matter is provided for producing superovulation in cattle comprising: an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone to luteinizing hormone is in a range of from 500 to 30,000.

The ratio described above may preferably be in a range of up to 3000; or of up to 2000; or of 2652 to 1; or of 2000 to 1655; or of 1000 to 1655; or of 1610 to 1.

Another broad aspect of this invention provides the use of a composition of matter comprising follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone to luteinizing hormone is in a range of from 500 to 30,000, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1, for the new use of producing superovulation in cattle. Such use may be by parenteral injection, e.g., the use of 75 units of the composition, more specifically in 8 equal doses at approximately 12 hour intervals, of the above-identified composition.

Thus, the present invention provides a hormone composition and method for producing an optimum superovulation response in cattle.

The composition of an aspect of the present invention resulted from the discoveries that high levels of progesterone at estrus reduce fertilization rates in cows, and that treatment of cows with commercially available FSH preparations (FSH-P) elevates blood progesterone levels during estrus. Accordingly, while FSH-P may induce many follicles to ovulate during superovulation, fertilization of these ova is reduced by the elevation of blood progesterone levels during estrus.

It was thought that LH contamination of FSH-P was one of the likely causes of the abnormally high preovulatory progesterone levels in cattle. To test this theory, LH was removed from FSH-P and cattle were treated with the FSH-rich preparation. Better

results were achieved with the FSH-rich preparation in terms of fertilization while the total ova produced decreased somewhat.

It was then determined that by including a small amount of LH in the FSH preparation the recruitment of follicles was increased as expressed by total embryos recovered. Accordingly, it was deduced that the amount of LH in the FSH preparation had to be optimized in order to maximize both follicle recruitment and fertilization rates during superovulation in cattle.

The composition of an aspect of the present invention, preferably, has a FSH/LH ratio of from 500 to 30,000 to maximize follicle recruitment and fertilization rates during superovulation in cattle. The composition of a preferred aspect of the present invention, most preferably, has a FSH/LH ratio of from 1000 to 1655.

The composition of one aspect of the present invention is, preferably, given to cattle by injection. A parenteral solution of the composition of aspects of the present invention is preferably prepared by forming a solution of the composition of the present with saline or phosphate buffered saline (PBS). The composition of aspects of the present invention is preferably given to cattle at a dose rate of 75 units (NIH-FSH-S1) by eight equal injections of 9.375 units over a period of 4 days at approximately 12 hour intervals.

In the accompanying drawings,

Figure 1 is a graphical representation of the chromatogram of commercially available FSH-P on QAE-Sephadex A-50. (SEPHADEX is the trade-mark of Pharmacia Fine Chemicals Inc. for synthetic organic compounds derived from the polysaccharide, dextran.)

The column dimensions were 5 x 33 cm. A sample load of 10 gm FSH-P was dissolved in 120 ml starting buffer (20 nM ammonium acetate, pH 7.2) and was applied to the column. The column was developed with 4.6 litres of starting buffer to elute the pLH in the sample (Peak A). Elution with 250 nM ammonium acetate was then begun and the pure FSH (FSH-W) fraction was collected (Peak C). The post-LH fraction is labelled peak B and the post-FSH is labelled peak fraction D.

The present invention will be described in more detail with reference to the following examples.

EXAMPLE 1

USE OF PORCINE FOLLICLE STIMULATING HORMONE AFTER CHROMATOGRAPHIC PURIFICATION IN SUPEROVULATION OF CATTLE

An FSH-rich fraction hereinafter referred to as (FSH-W), free of detectable LH, was used to superovulate cattle. Three experiments were conducted to determine the optimal dose and treatment regimen for FSH-W, to compare FSH-W and FSH-P (a commercial preparation available from Burns Biotec, Omaha, NE), and to study the effects of adding luteinizing hormone (LH) to the FSH-W.

Brahman crossbred cows were used in all experiments. The cattle were managed and superovulated in a similar manner as previously described in Donaldson, Theriogenology 21:517-524 (1984). The superovulation treatment was conducted over a period of 4 days and cows were treated twice daily at approximately 7 AM

and 6 PM each day. Estrus was controlled with prostaglandin $F_{2\alpha}$ (PGF), available from the Upjohn Co., Kalamazoo, MI) given in three doses of 35/15/15 mg morning, noon, and night on the third day, or with a cloprostenol dose (Estrumate, Haver Lockhart, Shawnee, KS) of 2.0 cc on the morning of the third day of superovulation as described in Donaldson, Theriogenology 21:1021-1022 (1984).

Estrus was monitored three times per day for about

45 minutes and the cows were bred about 6, 18 and 32 hours after detection of estrus. Embryos were collected nonsurgically and classified on the basis of microscopic appearance into transferable and nontransferable, unfertilized and fertilized degenerate (Donaldson, Theriogenology 21:517-524 (1984)). A transferable embryo appeared vital and was usually symmetrical and approximately round. The blastomeres were distinct with smooth membranes, without vesicles or excessive cellular debris beneath the zona pellucida. Non-transferable (degenerate) embryos had any or several of the following features: flattened, fuzzy membranes; grainy or dark appearance; cracked or broken zona. A fertilized degenerate embryo had clear evidence of cleavage whereas unfertilized eggs had perfectly spherical zonas containing single cells without evidence of cleavage. Embryo collection was performed on those cows that came into estrus. The data were analyzed by one- or two-way analysis of variance (ANOVA). Variance is represented by standard deviations of the mean.

The FSH-W preparation was produced from a commercially available porcine pituitary gland preparation (FSH-P, Lots 550C81 and 551C81 from Burns Biotec, Omaha, NE) by employing a QAE-A50 chromatography step procedure that separates pLH from pFSH (S.D. Glenn, unpublished). The FSH-W preparation can also be produced by the same chromatography step procedure from the pituitary glands of domestic animals such as sheep and pigs. First, however, the FSH must be removed from the pituitary gland. This process involves taking a pituitary gland from an animal and either fresh freezing it or lyophilizing it. If the pituitary is fresh frozen the water must be removed by acetone drying. Next, the acetone dried powder or freeze dried powder is extracted four times with a varying ratio of ethanol and tris buffer starting at 75% ethanol and working down to 20%. Each extract is then processed to remove the FSH and LH from the extract which is accomplished by cutting the extract with membranes by putting it through a 0.2 filter to remove all the fats, putting it through a 100,000 filter to remove all the proteins and other components

greater than 100,000 Daltons and finally putting it over a 10,000 Dalton membrane to concentrate the volume. The concentrated solution is then freeze dried or placed directly on the QAE-A50 column. The procedure exploits the differential affinity of LH and FSH in a low ionic strength buffer, e.g. 20 mM ammonium acetate at a pH of 7.2 to separate the LH and the FSH. The LH fraction contains some FSH activity, whereas the FSH fraction contains no detectable LH activity. The column elution is continued with 20 mM ammonium acetate at a pH of 7.2. After this step, the FSH activity retained on the column (approximately 66% of the total) is eluted with a 250 mM ammonium acetate buffer, pH 7.2. An inactive post-FSH fraction may be eluted with 500 mM ammonium acetate buffer, pH 7.2, if the remaining protein is to be accounted for.

With Lot 551C81, a 1.8-gm QAE-A50 column chromatography load was used. To obtain more material, the chromatography load was scaled up five-fold and a 10-gm load of Lot 565F82 was processed to obtain material of similar potency. The FSH and LH activity was assayed by a radioligand procedure as described previously by Bousfield et al., J. Biol. Chem., 259:1911-1921 (1984). The receptor assays used ^{125}I-labelled hCG or equine FSH as radioligands and rat testis (Moore et al., J. Biol.Chem. 255, 6930-6936 (1980)) or chicken testis homogenate (Glenn et al., Biol. Reprod. 24 (Suppl. 1) 117A Abstr. (1981)) as receptor preparations, respectively. The hormones were labelled to a specific

activity of 25 to 50 uCi/ug. Under these conditions, the assay is linear for porcine FSH in the range of 2 to 200 ng of pure porcine FSH. The LH radioligand assay is linear over the range of 10 to 1000 ng of pure porcine LH. All potency estimates were made from the parallel portion of the competitive binding curves for the unknown. Relative potencies were calculated from the ID_{50}s determined from the inhibition curves (Liu et al., J. Biol. Chem. 249:5544-5550 (1974)). Potency was expressed in terms of the NIH-LB-S16 reference preparation for LH and NIAMDD-OFSH-13 reference preparation for FSH. Potency is

expressed with these preparations equal to one unit by definition. The NIH-LH-S16 is essentially equipotent to the NIH-LH-S1 preparation. However, the NIAMDD-OFSH-13 is approximately 15 times the potency of the old NIH-FSH-S1 preparation. The potency estimates for the hormones used in the experiments are shown in Table 1.

TABLE 1

Separation and assay of FSH-W from FSH-P

Lot No. of FSH-P		551C81	565F82
FSH-P, starting wt., gm		1.8	10.0
FSH-W	gm	0.8313	3.485
LH fraction	gm	0.8097	0.8640
Post-FSH fraction[a]	gm		2.089
Recovery	%	91.1	64.5
Relative potencies[b]			
FSH-P	FSH	0.32	0.35
	LH	0.039	0.075
FSH-W	FSH	0.5	0.66
	LH	<0.005	<0.011

[a] An inactive fraction eluted after the FSH, with 500 mM ammonium acetate.

[b] Relative to National Institutues of Health standards NIAMDD-OFSH-13 and NIH-LH-S16.

The elution pattern for the material coming off the QAE-A50 column is typical and is depicted in Figure 1. The FSH-rich fraction (FSH-W) had a biological activity of 0.66 x NIAMDD-OFSH-13 with an undetectable LH potency (i.e., it was less than 0.011 x NIH-LH-S16, the limit of detection in the assay at the highest dose tested). The recovery of material from the columns was 91.1 and 64.5%.

Although the FSH-rich fractions are essentially free of LH, the FSH-W does not represent pure porcine FSH. Pure porcine FSH has a potency of about seven times NIAMDD-OFSH-13 (Closset et al., Eur. J. Biochem. 86:105-113 (1978); Whitley et al., Endocrinology 102: 1874-1886 (1978)). Thus, the fractions obtained here are about 11% pure but are suitable for the studies undertaken.

Experiment 1:

120 cows were used in a 3 x 2 factorial design to test the effects of dose rate of FSH-W and treatment regimen on embryo production. The dose rates used were 2.7, 5.4, and 10.8 units (NIAMDD-OFSH-13). These dose rates were

calculated from the potency estimates to be equipotent in FSH with 14, 28, and 56 mg (Armour units) FSH-P. The two treatment regimens consisted of eight individual injections of one-eighth of the total dose (constant regimen) or a descending dose of 19, 14, 10, and 7% of the total dose given twice a day for 4 days. Between 5 and 20 cows per week were assigned to experimental treatments. Treatments were assigned randomly to weeks and within weeks to each cow. Usually two treatments were assigned per week, but each treatment was represented within at least 2 wk.

The results of Experiment 1 are found in Table 2 below.

TABLE 2

Effects of FSH-W dose rate and treatment regimen on Mean Embryo Production (Experiment 1)

Treatment	Embryo Production (Mean ± SD) Number transferable	Total recovered	Percent transferable	Number fertilized
Dose x regimen				
2.7 units (NIH)a Constant	4.7 ± 3.9	9.1 ± 7.2	66 ± 36	6.2 ± 5.0
2.7 units Descending	4.2 ± 3.0	6.8 ± 4.5	67 ± 33	5.7 ± 3.6
5.4 units Constant	7.8 ± 7.3	15.4 ± 18.0	64 ± 32	10.1 ± 10.6
5.4 units Descending	6.4 ± 5.1	12.3 ± 8.1	51 ± 27	10.3 ± 6.7
10.8 units Constant	4.3 ± 4.8	10.1 ± 8.6	35 ± 25	6.6 ± 6.9
10.8 units Descending	1.8 ± 1.7	12.5 ± 8.8	19 ± 22	3.3 ± 3.1
Both regimens (constant and descending)				
2.7 units	4.5 ± 3.5	8.0 ± 6.2	66 ± 35	5.9 ± 4.4
5.4 units	7.0 ± 6.5	13.8 ± 14.6	57 ± 31	10.1 ± 9.1
10.8 units	3.1 ± 3.9	11.2 ± 8.8	27 ± 25	5.1 ± 5.8
All doses (2.7, 5.4, and 10.8 NIH units)				
Constant	5.6 ± 5.8	11.5 ± 12.6	56 ± 35	7.6 ± 8.1
Descending	4.3 ± 4.0	10.7 ± 7.9	46 ± 34	6.7 ± 5.5
BY ANOVA P = Dose	0.003	0.053	0.001	0.004
Regimen	0.126	0.678	0.089	0.652
Interaction	0.696	0.527	0.580	0.527

[a]National Institutes of Health.

There was a significant effect of dose of FSH-W on the number of transferable embryos recovered ($P = 0.003$). The number of transferable embryos increased from 4.5 ± 3.5 to 7.0 ± 6.5 and then decreased to 3.1 ± 3.9 with increasing dose. The total embryos recovered increased from 8.0 ± 6.2 to 13.8 ± 14.6 and then to 11.2 ± 8.8 ($P < 0.001$) with increasing dose, while the percent transferable declined

from 66 ± 35% to 57 ± 31% and then to 27 ± 25% (P = 0.001). These changes in the number and percent transferable were associated with changes in the number of fertilized embryos; this number increased from 5.9 ± 4.4 to 10.1 ± 9.1 and then declined to 5.1 ± 5.8 (P = 0.004). The number of fertilized embryos that degenerated was not affected by the dose of FSH-W (1.4 ± 2.5, 3.1 ± 3.7, and 2.0 ± 3.0, respectively; P=0.120). There were no significant interactions between dose and regimen.

Experiment 2

130 cows were used to compare FSH-W made from Lot 551C81 with FSH-P (Lot 551C81). The dose of FSH-W that gave the best embryo production response in the first experiment (5.4 mg) was compared with the dose of FSH-P (28 mg, Armour units) that was previously reported to give the best response (Donaldson, Theriogenology 22:205-212 (1984)). The cows were treated with a descending dose treatment regimen in which 19, 14, 10 and 7% of the total dose was given twice a day for 4 days. Cows were assigned randomly to the two treatments. Jugular blood samples were taken at the beginning of estrus for progesterone determinations in accordance with the procedure described by Reimers et al., J. Anim. Sci. 57:683-691 (1983), from 15 cows superovulated with FSH-P (28 mg) and from 24 cows superovulated with FSH-W (5.4 mg).

The results of Experiment 2 are found in Table 3 below:

TABLE 3

Comparison of FSH-W and FSH-P for the Superovulation of Cattle

Embryo Parameters	Treatment FSH-P Mean SD	FSH-W Mean SD	P
Number transferable	2.9 ± 4.0	6.3 ± 6.7	0.001
Total recovered	11.1 ± 10.0	12.1 ± 9.6	0.591
Percent transferable	30 ± 33	47 ± 35	0.007
Number fertilized	5.8 ± 6.7	9.0 ± 8.2	0.019
Number degenerate	2.4 ± 3.6	2.5 ± 3.0	0.819

When compared with 28 mg FSH-P, 5.4 units FSH-W significantly increased the number of transferable embryos from 2.9 to 6.3 (P = 0.001) without affecting the total embryos recovered (12.1 and 11.1, P = 0.591).

5.4 units of FSH-W was calculated to be equipotent with 28-mg equivalents of Armour units of FSH-P, and as noted above these dose levels have been found to be the most effective doses for both products. The percent transferable was higher in the FSH-W (47%) than in the FSH-P treated cows (30%, P = 0.007). This higher percentage resulted from an increase in the number of embryos fertilized from 5.8 to 9.0 (P = 0.019).

The blood progesterone levels (ng/ml) during estrus in the 15 cows treated with FSH-P (0.88 ± 0.69) were significantly higher (P=0.016) than in the 24 cows treated with FSH-W (0.45 ± 0.36). Normal blood progesterone levels in the cow during estrus range from 0.2 to 0.5 ng/ml (Lemon et al., J. Reprod. Fertil. 31:501-502 (1972)).

Crisman et al. (Theriogenology 15:141-154 (1980)) showed that excess progesterone (but not estradiol) increased ovum transport rates in the cow. The higher progesterone levels in the FSH-P treated cows may be caused

by the LH contamination, which enhances progesterone production in the theca interna of preantral follicles (Terranova et al., Biol. Reprod. 29:630-636 (1983)) or which luteinizes large follicles that subsequently produce progesterone. FSH can stimulate progesterone production in follicles itself (Lischinsky et al., Endocrinology 113:1999-2003 (1983)) and this progesterone production may be involved in the reduction in the number of fertilized embryos with higher doses of FSH-W. Previously, high blood progesterone levels at estrus have been associated with decreased embryo production in cattle (Greve et al., Theriogenology 21:238 Abstr. (1984).

Experiment 3:

50 cows were used in a 2 x 2 factorial design to test the effects on embryo production of adding LH (made from Lot 551C81 as described above) to the FSH-W preparation on the first day of FSH treatment to induce superovulation. The two dose levels of FSH-W were 5.4 and 8.3 units given in a constant regimen as in Experiment 1. LH was injected at the time of the two FSH-W injections at dose rates of 0 or one mg NIH-LH-S16. This LH included 0.06 units of FSH per injection as a contaminant. Cows were assigned randomly to the four treatments.

The results of Experiment 3 are found in Table 4 below:

TABLE 4

Effect of adding LH to FSH-W on Mean Embryo Production

Treatment	Embryo Production (Mean ± SD) Number transferable	Total recovered	Percent transferable	Number fertilized
Dose x LH				
5.4 units (NIH)a + LH	5.1 ± 3.2	10.8 ± 4.6	55 ± 34	7.6 ± 3.4
5.4 units NO LH	7.8 ± 4.4	13.4 ± 5.5	60 ± 28	11.1 ± 5.3
8.3 units + LH	3.6 ± 6.3	10.8 ± 7.8	41 ± 37	2.7 ± 1.7
8.3 units NO LH	7.8 ± 7.7	16.7 ± 9.9	45 ± 28	14.7 ± 10.1
Both LH treatments				
5.4 units	6.4 ± 4.1	12.1 ± 5.2	55 ± 31	9.3 ± 4.8
8.3 units	5.8 ± 7.4	13.9 ± 9.4	43 ± 33	8.7 ± 9.4
Both doses				
LH	4.4 ± 5.0	10.8 ± 6.3	48 ± 36	5.3 ± 3.6
NO LH	7.8 ± 6.3	15.0 ± 8.1	53 ± 29	12.8 ± 8.1
ANOVA P = LH	0.052	0.06	0.635	0.001
Dose	0.698	0.525	0.126	0.742
Interaction	0.665	0.529	0.945	0.024

[a]National Institutes of Health.

Adding LH to the FSH-W on the first day of FSH treatment reversed the effect of removing LH from FSH-P. The number of transferable embryos were reduced from 7.8 ± 6.3 to 4.4 ± 5.0 (P = 0.05, Table 4). Total embryos recovered was reduced (P = 0.06), and percent transferable was not significantly different (P = 0.635). The number of fertilized embryos was reduced from 12.8 ± 8.1 to 5.3 ± 3.6 by the addition of LH dose to FSH-W (P < 0.001). There was a significant interaction (P = 0.024); LH had more effect on fertilization at the higher dose. Added LH significantly reduced the number of fertilized degenerating embryos (4.8 ± 3.9 to 1.5 ± 1.6, P = 0.001), but the percent degenerate (31 ± 31 and 38 ± 28%, P = 0.55) remained the same because there

was a parallel reduction in the number of fertilized embryos.

The effect of altering the LH content of the FSH on total embryo production was not clear, but embryo recovery is not a sensitive measure of ovulation rate because only about 40% of the ovulations are represented by embryos recovered (Donaldson, Vet. Rec. 117:33-34, 1985).

In cattle superovulated with FSH-P, fertilization rates have been found not to be improved by increasing the number of times a superovulated cow was bred above two times or by increasing the number of straws of semen used at each breeding above one (Critser et al., Theriogenology 13:397-405 (1980); Donaldson, Vet. Rec. 117:35-37 (1985). Based on these results, it was hypothesized that there are factors other than sperm numbers that interfere with fertilization. In the normal estrus cycle, the LH surge triggers the maturation phase of the oocyte, establishing the time frame for fertilization (Moor et al., Theriogenology 21:103-116 (1984)). Excess LH in a superovulatory hormone causes premature stimulation of the oocyte (Moor et al., Theriogenology 21:103-116 (1984)) so that the oocyte may not be capable of being fertilized at the normal time. In ewes, superovulation reduces sperm transport (Armstrong et al., Proc. 10th Inter. Cong. Anim. Reprod. Art. Insem., Urbana, IL, 1984, pp. VII-8-VII-15). In rats, oocytes produced after superovulation with pregnant mare serum gonadotropin are normal (Evans et al., J. Reprod. Fertil. 70:131-135 (1984)) but have reduced fertility due to complete or partial failure of fertilization (Walton et al., J. Reprod. Fertil. 67:91-96 (1983); J. Reprod. Fertil. 67:309-314 (1983)). Therefore, the demonstrated increase in blood progesterone levels at estrus offers several mechanisms whereby fertilization rates could be influenced by the levels of LH and FSH in the superovulation treatments namely, increased tubal transport of ova, decreased sperm capacitation, or decreased sperm transport. LH in superovulation regimens appears to be deleterious and exerts its effect at several stages in the reproductive process. Excluding LH from an FSH preparation for

superovulating cattle increased the production of transferable embryos by increasing the number of fertilized embryos.

EXAMPLE 2

DOSE RESPONSE TO FSH-W

WITH AND WITHOUT LH CONTAMINATION

FSH-P (lot 565F82, Burns Biotec, Omaha NE) was separated into an FSH-W fraction without any detectable LH using sephadex QAE-A50 column chromatography as described in Example 1. The relative potency of FSH and LH for the FSH-P (0.32 and 0.039) and FSH-W (0.66 and < 0.01) fractions to NIH-FSH-S13 and NIH-LH-S16 were determined using chicken and rat testicular homogenate receptor assays. The assays indicated that 5.4 units FSH-W was approximately equipotent in FSH to 20 Armour units (often expressed as mg) FSH-P. The dose response to FSH-P was measured in 80 Brahman cross cows (20 per dose level) and to FSH-W in 140 Brahman cross cows (37, 36, 27, 31 and 9 per dose within increasing doses respectively).

The results of this study appear in Table 5 below.

TABLE 5

FSH-W				FSH-P			
	MEAN EMBRYOS/COW				MEAN EMBRYOS/COW		
FSH units	#trans-ferable	total	%trans-ferable	Armour units	#trans-ferable	total	%trans-ferable
2.7	4.5	8	66				
5.4	7.1	14	57	20	2.1	2.6	72
8.3	4.9	11	46	28	3.9	10.1	47
10.8	3.4	11.2	29	40	2.5	8.2	35
16.2	3.5	9.4	35	60	0.9	6.3	16
By ANOVA within columns							
P =	0.043	0.161	0.000		0.006	0.003	0.000

The removal of the LH increased the effectiveness of the FSH-W by lowering the dose giving the maximum response, and by apparently increasing the number of transferable embryos produced at that response. The decline in embryo production beyond the most effective dose was not as large with FSH-W as it was with FSH-P. Removal of the LH from the FSH-P altered the shape of the dose response curve, and increased the responsiveness of the cow to FSH.

EXAMPLE 3

EFFECTS OF LH ON EMBRYO PRODUCTION IN SUPEROVULATED COWS

As noted above, it has been shown that normal preovulatory progesterone (P4), LH and FSH concentrations are necessary for optimal embryo production from superovulated cows. Abnormal concentrations of P4, LH and FSH are followed by abnormal follicular/oocyte maturation and lowered embryo production. Since LH contamination of FSH preparations is thought to be one of the likely causes of abnormal preovulatory progesterone concentrations, this example was designed to study the effects of LH added to FSH on embryo production in the cow.

In this study, three FSH preparations were used, FSH-P (available from Burns Biotec Omaha NE), FSH-W and FSH-S. FSH-W was produced as described in Example 1 from FSH-P or from porcine pituitaries and contained no detectable LH. The FSH and LH activity of the FSH-W and three of six lots of FSH-P used in this study were assayed by a radioligand receptor assay using ^{125}I-labelled HCG or equine FSH as radioligands (Bousfield et al., J. Biol. Chem.

259, 1911 (1984)) and rat testis (Moore et al., J. Biol. Chem. 255, 6930 (1980)) or chicken testis (Glenn et al., Biol. Reprod. 24 (Suppl. 1) 117A (1981)) homogenates as receptor preparations respectively. Potency was expressed in terms of NIH-LH-S1 and NIH-FSH-S1 preparations. FSH/LH ratios were calculated in terms of these units. FSH-S was made from FSH-W by adding an aliquot of FSH-P to achieve an FSH content per dose of 75 units with an FSH/LH of greater than 500 and less than 2000.

A total of 273 cows were superovulated at seven embryo transfer centers. At each of the centers FSH-W was substituted for the FSH-P that was in normal use. At two centers FSH-S was also substituted. These superovulations using FSH-W and FSH-S were compared with contemporary controls receiving FSH-P. Cows from a wide range of cattle breeds (both beef and dairy) were superovulated with 75 or 112 units FSH-W, FSH-S or FSH-P (28-42 mg Armour units). Five centers used 75 units and two centers used 112 units. The total number of embryos and ova, the number of transferable embryos, the number fertilized and the number of fertilized degenerates were recorded (Donaldson Vet. Rec. 117, 35 (1985). Superovulation techniques varied from center to center but generally followed the non-surgical technique described by Elsden et al., Theriogenology 6, 523 (1976).

The data were collected over an 18 month period and no efforts were made to detect or correct for variations in techniques between centers. It was assumed that the FSH-P preparations used and not assayed had similar FSH/LH ratios to the lots that were assayed. The ratios of percent transferable, percent fertilized and percent fertilized degenerate were calculated for each observation. The data were analyzed by analysis of variance and Student's two tailed t test between centers and then pooled over all centers and both FSH doses.

The results of this study appear in Table 6 below.

TABLE 6

EFFECT OF LH ON EMBRYO PRODUCTION
IN SUPEROVULATED COWS (MEANS +S.D.)

	FSH-W	FSH-S	FSH-P	P<
FSH/LH	> 20,000	>500	<100	
# COWS	94	89	90	
EMBRYOS				
TOTAL	8.8 + 7.4	10.6 + 9.2	8.1 + 7.2	0.108
FERTILIZED	7.6 + 7.3	9.0 + 8.5	6.0 + 6.6	0.040
TRANSFERABLE	5.7 + 5.8	5.8 + 6.4	3.3 + 4.7	0.006
%TRANSFER-ABLE	66 + 33	51 + 35	37 + 38	0.001
% FERTILIZED	83 + 28	81 + 29	62 + 42	0.001
% FERTILIZED DEGENERATE	21 + 27	34 + 30	39 + 37	0.002

There was no statistical difference in embryo production between centers within FSH preparations and between dose rates, so the data was pooled. FSH-S produced an average of 10.6 embryos and ova per flush which was not significantly different from the 8.8 and 8.1 produced by FSH-W and FSH-P ($P < 0.108$). The addition of LH below the FSH/LH of 500 significantly reduced the number of embryos of transferable quality from 5.7 (FSH-W), and 5.8 (FSH-S) to 3.3 (FSH-P, $P < 0.006$). As LH levels increased the transferable percent declined from 66% (FSH-W), to 51% (FSH-S) and 37% (FSH-P, $P < 0.001$) because of changes in the number of embryos fertilized and an increase in the percent of fertilized embryos that degenerated. The number of fertilized embryos increased from 7.6 (FSH-W) to 9.0 (FSH-S, $P > 0.01$) and then declined significantly to 6.0 with FSH-P ($P < 0.04$). The percentage of fertilized embryos that degenerated increased from 21% (FSH-W) to 34% (FSH-S, $P < 0.032$) and then to 39% (FSH-P, $P < 0.002$) as LH levels in the FSH preparations increased.

The precision of this study may have been reduced

because of the involvement of seven different embryo transfer centers, but the results suggest important differences exist between the superovulation response produced by these three hormone preparations, which differed only in their LH content.

The dose rates for each FSH preparation were selected to be equipotent in FSH and to be optimum for transferable embryo production (Donaldson, Theriogenology 22:205 (1984); Donaldson et al., Theriogenology 23, 189 (1985)). LH contamination of FSH reduced the fertilization rates of ova produced by superovulation. LH appears to specifically block fertilization, the mechanism for which may be through premature stimulation of the maturing oocyte (Moor et al., Theriogenology 21, 103 (1984)) so that the oocyte is not capable of being fertilized. This is supported by an earlier study that showed that fertilization problems in superovulated cows cannot be overcome by multiple inseminations with many doses of semen (Donaldson, Vet. Rec. 117, 35 (1985)). The number of times that the cows were bred and the quantity of semen used was not standardized in this experiment, but the routine practice at all but one of the embryo transfer centers was to breed superovulated cows at least twice with a total of at least two doses of semen. The slight increase in the number of fertilized embryos in the FSH-S group was offset by the increase in the degeneration of fertilized embryos with increasing LH levels. The mechanism by which fertilized embryos degenerate with increasing LH levels is not known. As the LH content of the FSH increased, the variability of some of the responses increased as measured by the standard deviation of the mean. This was seen in the number and percentage transferable, and the number and percentage fertilized. Thus, the variability of superovulation response may be reduced by controlling the LH levels in the superovulatory hormones.

There are problems comparing the assay results from different laboratories of various gonadotropin preparations used in the superovulation of cattle because of the variety of assays and standards used. It appears that all FSH-P

preparations have an FSH/LH of less than 100. These results confirm the conclusions drawn from endocrine data (Donaldson, Theriogenology 23, 441 (1985) and Calleson et al., Theriogenology 25, 71 (1986)) that the traditional superovulatory treatments with gonadotropins containing LH disturbs the normal oocyte and follicular development leading to oocytes of inferior quality; and that FSH is mainly responsible for the number of embryos and ova produced and LH for their subsequent quality.

EXAMPLE 4

LH EFFECTS ON SUPEROVULATION AND FERTILIZATION RATES

The effects of LH during superovulation and at the subsequent estrus were studied in 108 beef and dairy cows. The experimental design was a 3x2 factorial with three FSH preparations, having different concentrations of LH, and two levels of LH (10 and 0 units) injected six hours after the onset of estrus. The FSH preparations were FSH-W, FSH-S and FSH-P. The FSH-W and FSH-S were prepared from FSH-P (Armour Pharmaceutical Co., Chicago, IL) as described in Examples 1 and 3, respectively.

The hormones were assayed by radioligand receptor assay referenced to the NIH-FSH-S1 and NIH-LH-S1 standards. The FSH/LH ratios of the three FSH preparations were in the following ranges 30,000 (FSH-W), 1600 (FSH-S) and 114 (FSH-P).

The cows were superovulated with 75 units of FSH divided into eight equal doses administered at 12 hour intervals for four days starting in the evening. Chlorprostenol 2cc (Estrumate, Haver-Lockart, Shawnee, KS) was given at the time of the fifth FSH injection. The cows were observed closely for the onset of estrus, and half of them received 10 units LH six hours later. Cows were bred with one straw of frozen semen four to 22 hours after the onset of estrus. Embryos were recovered nonsurgically seven days later and the total, number transferable and the number fertilized recorded. Data were analyzed by two way analysis of variance.

Transferable/total embryos recovered were 2.4/4 (FSH-W), 6/10.5 (FSH-S) and 1.9/5.4 (FSH-P). Total and

transferable embryos were significantly different (P=0.011 and 0.014). The percent transferable was lower in the FSH-P (35%) than in the other groups (55% and 52%, P=0.047). The LH effect was in the percent fertilized, being 84% (FSH-S), 80% (FSH-W) and 48% in the FSH-P group (P=0.001). LH at estrus did not affect transferable or total embryos (3.5/7 with LH and 4.9/8.5 in controls, P=0.667 and 0.756). The percent of ova fertilized in the LH at estrus group (70%) tended to be lower than the controls (82%, P=0.08), as did the percent transferable (43% versus 56%, P=0.153). Embryo production was significantly affected by the LH levels in the FSH but not by the LH injected at estrus. High levels of LH in the FSH reduced fertilization rates. Low fertilization rates have traditionally been approached by increasing the number of doses of semen used and the number of times a donor cow is bred. In this study one breeding with one dose of semen produced normal fertilization rates when the level of LH in the FSH hormone was reduced, indicating that low fertilization rates with FSH-P are specifically an LH problem not a semen quantity or a number of times bred problem.

EXAMPLE 5

FIELD TESTS WITH THREE FSH PREPARATIONS

The three FSH preparations were used almost exclusively on problem donors that had failed to respond to commercially available Armour FSH-P. Each embryo transfer center testing the preparations were asked to report contemporary results with FSH-P. The data are therefore heterogeneous and may be best used to demonstrate the type of results in independent hands and fertilization rates. The actual numbers and differences between treatments have to be interpreted with caution.

Three hormone preparations were tested each with a different formulation. They were:

FSH-W batches 200 and 167 of an FSH preparation containing no detectable LH or having a ratio of FSH/LH of 30,000.

FSH-S, an FSH preparation according to the present invention containing some LH having an FSH/LH ratio of 1610.

FSH-P is a commercial FSH preparation containing much LH having an FSH/LH ratio of <114.

The FSH-W and FSH-S preparations utilized in this study were prepared from FSH-P (Armour Pharmaceutical Co., Chicago, IL) as described in Examples 1 and 3, respectively.

The data represents most breeds. Six of the 10 embryo transfer centers only used FSH-W on problem cows and the production from these cows was less than with FSH-P. The others used FSH-W on the normal run of cows and they had production equal to or better than FSH-P. There were obvious differences between centers on classification of embryos into unfertilized and fertilized degenerate. No attempt has been made to correct data for any of these differences. Each center used at least 2 FSH products.

The data has been divided on the basis of product and dose. FSH-W and FSH-S have been measured in terms of the NIH-FSH-FSH1 standard. The three doses are 75, 112 and 150 units. (75 units is the same as 5 units S13, the units in which batch 200 was measured). The equivalent Armour units are 28, 42 and 56. Collections where no embryos were recovered are not included in this analysis, mainly because there seemed to be large irregularities in the way they were reported. It is not believed that the majority of zero collections have anything to do with the FSH, and their absence does not affect the fertilization and degeneration rates that were the focus of this example.

The data confirms the controlled experiments that FSH-W and FSH-S improved fertilization rates. This effect carries over into percent transferable, and in the case of FSH-S into the number transferable. FSH-S increased recruitment of follicles over FSH-W as expressed by the total embryos recovered. There is an indication that increasing the dose of FSH reduces the degeneration rate.

TABLE 7

EMBRYOS
TRANSFERABLE/TOTAL
(% TRANSFERABLE)

PREPARATION	DOSE LEVEL 75	112	150	TOTAL
FSH-W	4.8/9.4 (55)	4.7/7.2 (70)	5.4/8 (62)	4.9/8.9 (58)
# FLUSHES	158	26	46	229
FSH-S	7.5/11.4 (64)	7/15.1 (46)	8/11.5 (71)	7.5/12.2 (62)
# FLUSHES	34	11	12	57
FSH-P	5.2/11.4 (48)	4/8.8 (55)	6.3/10.1 (54)	5.4/10.8 (50)
# FLUSHES	157	26	60	243
TOTAL	5.2/10.5 (53)	5.0/9.5 (59)	4.5/9.4 (60)	5.3/10.1 (55)
# FLUSHES	349	63	117	529

P VALUES DOSE 0.566, 0.081, 0.549
PREPARATION 0.029, 0.087, 0.138

TABLE 8

EMBRYOS
FERTILIZED, % FERTILIZED

PREPARATION	DOSE LEVEL 75	112	150	TOTAL
FSH-W	6.4,73	5.8,80	6.8,82	6.4,75
FSH-S	10.4,95	9.9,91	11,88	10.5,92
FSH-P	7.2,65	5,65	7.2,63	7,64
TOTAL	7.2,71	7.4,73	6.2,76	7.1,72
P VALUES DOSE			0.586,	0.966
PREPARATION			0.000,	0.000

TABLE 9
EMBRYOS
FERTILIZED DEGENERATE, % FERT. DEGENERATE

PREPARATION	DOSE LEVEL 75	112	150	TOTAL
FSH-W	1.9,26	1.3,20	1.2,21	1.7,24
FSH-S	3.2,31	5.2,42	2.6,20	3.5,31
FSH-P	2.6,30	1,16	1,13	2,24
TOTAL	2.4,28	1.9,22	1.3,17	2.1,25

P VALUES DOSE 0.586, 0.966
PREPARATION 0.000, 0.000

EXAMPLE 6

This example compares a batch of FSH-S having a FSH/LH ratio of approximately 1000 and a batch of FSH-P having a FSH/LH ratio of approximately 114. The batch of FSH-S was prepared in accordance with the procedure described in Example 3.

The results of this comparison are shown in Table 10 below.

TABLE 10

	# Good Embryos Mean ± SD	Total Embryos Mean ± SD	% Good Embryos Mean ± SD
FSH-S	7.56 ± 6.98	14.2 ± 10.12	53.28 ± 31.64
FSH-P	4.39 ± 5.02	10.66 ± 7.82	42.56 ± 35.07

This example demonstrates that a reduction in LH content of the FSH preparation is beneficial in terms of the number of good embryos, the total embryos and percent good embryos.

EXAMPLE 7

DURATION-EFFICACY STUDY

Objective. The objective of this study was to determine the optimum duration of treatment with FSH-S.

Cows were selected to go on experiment after they had been detected in heat with a normal estrus interval (16 to 24 days). Cows were put into experimental groups randomly on the basis of their estrus dates. On a weekly basis all the ear tag numbers of the experimental cows that had been in estrus during the preceeding week were written on a separate card and the cards were selected at random and allocated to successive groups within breeds. The cows were then scheduled for starting on treatment on a day convenient for the subsequent collection date.

Cows were treated for 3, 4 or 5 days with 18.75 units of FSH-S per day in equal divided doses morning and evening. This is the same rate as a total dose of 75 units over 4 days. The FSH-S used in this study was prepared in accordance with the procedure described in Example 3. Three separate batches of FSH-S were used to treat the cows for this study. These batches had FSH/LH ratios of 1500, 1443 and 1267, respectively.

Embryo production was measured in terms of total embryos and ova, and the number of transferable embryos.

The results of this study are shown in Tables 11 and 12 below.

TABLE 11

Days of treatment with FSH	Transferable Embryos Mean ± SD	Total Ova + Embryos Mean ± SD
3	1.250± 1.699	3.950± 4.295
4	5.950± 4.421	1.050± 7.074
5	5.550± 5.903	8.200± 5.836

TABLE 12

Breed	Transferable Embryos Mean ± SD	Total Ova + Embryos Mean ± SD
Holstein	5.036 4.851	7.536 4.917
Beef	3.563 4.763	7.906 7.670

There was no breed effect observed for either parameter measured. Treatment for 4 and 5 days produced more transferable and total embryos than did treatment for 3 days. Treatment for 4 days appeared to give the best overall results.

EXAMPLE 8

This example demonstrates a comparison of equipotent batches of FSH-W having a FSH/LH ratio of 30,000 and commercially available FSH-P having a FSH/LH ratio of 114. The FSH-W preparation was produced in accordance with the procedure described in Example 3.

The results are shown in Table 13 below.

TABLE 13

Embryos	FSH-P	FSH-W	P
# transferable	2.92	6.32	0.001
total	11.12	12.05	0.591
% transferable	30.02	46.87	0.007
# fertilized	5.78	9.02	0.019
% fertilized	54.69	63.2	0.237
# degenerate	2.39	2.52	0.819
% degenerate	23.12	17.7	0.258

Cows superovulated with FSH-W performed significantly better in terms of # transferable embryos, % transferable embryos and # fertilized embryos. These results corroborate the results of Example 1.

EXAMPLE 9

DOSE RESPONSE STUDY

The objective of this study was to establish the optimum dose for a FSH-S preparation having a FSH/LH ratio of 1610. The FSH-S preparation utilized in this study was prepared in accordance with the procedure described in Example 3.

Cows were selected to go on experiment after they had been detected in heat with a normal estrus interval (16 to 24 days). Cows were put into experimental groups randomly on the basis of their estrus dates. On a weekly basis all the ear tag numbers of the experimental cows that had been in estrus during the preceeding week were written on a separate card and the cards were selected at random and allocated to successive groups within breeds. The cows were then scheduled for starting on treatment on a day convenient for the subsequent collection date.

Sixty cows (24 Holstein and 36 beef) were superovulated with 37.5, 75 or 150 units of FSH-S (20 per treatment). The results of this study appear in Table 14 below. In another experiment 45 cows (all beef) were superovulated with 75, 112 or 150 units of FSH-S. The results of this study appear in Table 15 below.

The total embryos and ova and the number of transferable embryos were recorded.

TABLE 14

Dose Rate	N	Transferable Embryos Mean ± SD	Total Ova + Embryos Mean ± SD
37.5	20	1.850 ± 2.128	3.700 ± 3.770
75	20	6.000 ± 5.060	10.200 ± 5.913
150	20	5.300 ± 3.621	9.250 ± 3.300

TABLE 15

Dose Rate	N	Transferable Embryos Mean ± SD	Total Ova + Embryos Mean ± SD
75	15	6.067 ± 5.431	7.667 ± 5.802
112	15	5.667 ± 3.200	9.800 ± 6.190
150	15	5.067 ± 4.061	8.467 ± 7.396

As shown in Table 14, the 75 and 150 units FSH-S produced significantly more transferable and total embryos than did 37.5 units. Also, as shown in Table 14, there was no difference between beef and dairy cattle. As shown in Table 15, there was no significant difference in embryo production between 75, 112 or 150 units. It was concluded that 75 units was the optimum dose rate.

EXAMPLE 10

This example is a dose-rate study for a batch of FSH-S having a FSH/LH ratio of approximately 1655. The FSH-S was prepared in accordance with the procedure described in Example 3.

The results are shown in Table 16 below.

TABLE 16

Dose Rate	Transferable Embryos Mean ± SD	Total Ova + Embryos Mean ± SD
75	8.29 ± 7.2	12.14 ± 8.41
112	2 ± 1.29	8 ± 8.33
150	1 ± 1.22	4.2 ± 4.92

This example demonstrates that a dose of 75 units of FSH-S gives optimum results in terms of both transferable embryos and total embryos and corroborates the results of Example 9.

EXAMPLE 11

This example demonstrates a study investigating dose and regimen effects of a FSH-W preparation having an FSH/LH ratio of 30,000. The FSH-W preparation was produced in accordance with the procedure described in Example 1.

The results are shown in Table 17 below.

TABLE 17

	Regime	Dose mg FSH (5.4 mg=75 units) 2.7	5.4	10.8	Total
# Good Embryos Collected	level	4.1	5.7	3.4	4.7
	descending	4.2	6.3	1.8	4.4
	Total	4.2	6.0	2.6	
Total Embryos Collected	level	6.9	10.5	9.1	9.0
	descending	6.8	12.7	13.4	10.5
	Total	6.8	11.4	11.5	
% Good Embryos Collected	level	59.3	59.3	23.9	53.2
	descending	66.8	48.7	18.8	48.5
	Total	63.4	54.6	21.3	
# Fertilized	level	5.8	7.9	6.1	6.9
	descending	5.6	10.2	3.4	6.7
	Total	5.7	8.9	4.5	
% Fertilized	level	78.8	79.4	41.6	72.6
	descending	85.7	75.3	27.5	67.3
	Total	82.7	77.6	32.9	
# Degenerate Embryos	level	1.4	2.0	1.9	1.8
	descending	1.5	3.6	1.6	2.3
	Total	1.5	2.7	1.7	
% Degenerate Embryos Collected	level	15.0	18.1	11.1	15.9
	descending	18.8	23.0	7.7	17.5
	Total	17.1	20.2	9.0	

The study demonstrated that level and descending dose regimes produced approximately equal results. The

study also demonstrated there is a significant dose effect with respect to the following criteria: number of good embryos collected, total embryos collected, number fertilized and % fertilized. Finally, the study demonstrated there was not a significant dose effect with respect to the following criteria: number of degenerate embryos and % degenerate embryos.

EXAMPLE 12

This study was a direct comparison of cows treated with FSH-P having an FSH/LH ratio of 114 and cows treated with FSH-S having an FSH/LH ratio of 504. The FSH-S used in this study was prepared in accordance with the procedure described in Example 3.

The results of this study are found in Table 18 below.

TABLE 18

	FSH-P	FSH-S
# Good embryos	0.69	5.80
Total embryos	7.60	8.70
% Good embryos	9.08	66.67

This study indicated that significantly better results in terms of number of good embryos and percent good embryos are achieved when cows are treated with FSH-S rather than FSH-P.

SUPPLEMENTARY DISCLOSURE

The composition for producing superovulation set out in the original disclosure, has now been found to be useful for mammals in general. Thus, the present invention as now provided by the present Supplementary Disclosure provides a hormone composition for producing superovulation in mammals. The composition has a particular ratio of follicle stimulating hormone (FSH) and luteinizing hormone (LH) which produces an optimum ovulation response in mammals and promotes out of season breeding an twinning. The composition can be produced from mammal pituitary glands or by recombinant DNA procedures and can be preserved in a phosphate buffered saline solution of thymol.

In another aspect, the present invention provides a composition and method for producing an optimum ovulation response in mammals.

It is an established commercial practice to stimulate estrus and ovulation in sheep, rabbits and other mammals at a time other than their normal breeding season or period. It is also a commercial husbandry practice to induce twinning in cattle and increase the numbers of offspring in sheep, pigs, and other species.

The present invention, as now provided by one aspect of the present Supplementary Disclosure, provides a composition of matter for producing superovulation in goats comprising: an effective amount of follicle stimulation hormone and luteinizing

hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range of from 500 to 30,000 to 1 or greater.

The present invention, as now provided by another aspect of the present Supplementary Disclosure, provides a composition of matter for producing superovulation in swine comprising: an effective amount of follicle stimulation hormone and luteinizing hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range of from 500 to 30,000 to 1 or greater.

The present invention, as now provided by yet another aspect of the present Supplementary Disclosure, provides a composition of matter for producing superovulation in sheep comprising: an effective amount of follicle stimulation hormone and luteinizing hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range of from 500 to 30,000 to 1 or greater.

The present invention, as now provided by still another aspect of the present Supplementary Disclosure, provides a composition of matter for producing superovulation in horses comprising: an effective amount of follicle stimulation hormone and luteinizing hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range of from 500 to 30,000 to 1 or greater, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1.

The present invention, as now provided by still another aspect of the present Supplementary Disclosure, provides a composition of matter for producing superovulation in humans comprising: an effective amount of follicle stimulation hormone and luteinizing hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range from 500 to 30,000 to 1 or greater, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1.

The present invention, as now provided by yet another aspect of the present Supplementary Disclosure, provides a composition of matter for producing superovulation in exotic mammals comprising: an effective amount of follicle stimulation hormone and luteinizing hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range from 500 to 30,000 to 1 or greater, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1.

The present invention, as now provided by still another aspect of the present Supplementary Disclosure, provides a pharmaceutical composition for producing superovulation in mammals comprising: an effective amount of follicle stimulation hormone and luteinizing hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range from 500 to 30,000 to 1 or greater, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in

the ratio of 2652 to 1, or in the ratio of 1610 to 1; the pharmaceutical composition being in an aqueous solution with saline or phosphate buffered saline and an antimicrobial preservative which is compatible with the pharmaceutical composition.

The antimicrobial preservative preferably is comprised of 5-methyl-2(1-methylethyl)phenol, e.g. at least 0.04% by weight or greater, or preferably from 0.5% by weight up to 0.1% by weight of the pharmaceutical composition solution. In another variant thereof, the ratio of follicle stimulating hormone to luteinizing hormone is in a range from 1000 to 23,000 to 1, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1.

The present invention, as now provided by still another aspect of the present Supplementary Disclosure, provides an injectable pharmaceutical composition for producing superovulation in mammals comprising: an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range of from 500 to 30,000 to 1 or greater, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1; the injectable pharmaceutical composition being in solution with saline or phosphate buffered saline and an antimicrobial preservative which is compatible with the injectable pharmaceutical composition.

The antimicrobial preservative comprises at least 0.04% by weight of the solution, preferably 0.04 to 0.1% by weight of the solution. The antimicrobial preservative is preferably comprised of 5-methyl-2-(1-methylethyl)phenol.

The present invention, as now provided by yet another aspect of the present Supplementary Disclosure, provides a composition of matter for producing superovulation in mammals comprising: an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone to luteinizing hormone is in a range of from 500 to 50,000 to 1, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1, wherein, on a molecular weight basis, the luteinizing hormone is present at 16% by weight at the 500 to 1 ratio and 0.16% by weight at the 50,000 to 1 ratio.

The present invention, as now provided by yet another aspect of the present Supplementary Disclosure, provides the use of a composition comprising follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone to luteinizing hormone is a range of from 500 to 30,000 to 1 or greater or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1, for the new use of producing superovulation in mammals, e.g. by parenteral injection, e.g., 8 equal doses at approximately 12 hour intervals.

The present invention, as now provided by still another aspect of the present Supplementary Disclosure, provides the use of a composition of matter comprising an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone to luteinizing hormone is in a range of from 500 to 30,000 to 1 or greater, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1, for the new use of achieving out of season breeding for mammals characterized as having breeding seasons.

The present invention, as now provided by yet another aspect of the present Supplementary Disclosure, provides the use of a composition of matter comprising an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulation hormone to luteinizing hormone is in a range of from 500 to 30,000 to 1 or greater, or in the range of up to 3000, or in the range of up to 2000, or in the range of 2000 to 1655, or in the ratio of 2652 to 1, or in the ratio of 1610 to 1, for the new use of enhancing twinning in sheep.

The present invention, as now provided by still another aspect of the present Supplementary Disclosure, provides the use of a composition of matter comprising an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone to luteinizing hormone is in a range of from 500 to 30,000 to 1 or greater, for the new use of enhancing twinning in cattle.

In the further drawings accompanying the present Supplementary Disclosure,

Figures 2 through 7 illustrate percent total specific binding plotted against log dose for each FSH batch in phosphate buffered saline (PBS) with and without Thymol comparing zero days, four days, and the NIH-FSH-S8 standard. The curves are nearly identical in each case, indicating that there has been no change in FSH activity by placing in the PBS solution 1% by weight Thymol.

Figures 2 and 3 are related since they were prepared using the same batch, with Figure 2 illustrating

the batch with 0.1% by weight Thymol preservative and Figure 3 illustrating the batch without Thymol preservative added. Figures 4 and 5 illustrate a different batch with Figure 4 having 0.1% Thymol preservative added and Figure 5 without any Thymol preservative addition. Figures 6 and 7 present yet another batch with Figure 6 including 0.1% by weight Thymol preservative and Figure 7 presenting data without the preservative addition.

Super ovulation is used in many species of mammals using the same drug for a number of husbandry practices. Superovulation for embryo collection and transfer is used in sheep, cattle, swine, goats, horses, and many zoo mammals. Superovulation for production of multiple ovine which are allowed to develop is used for twinning in cattle and sheep and for out of season breeding in sheep. The physiological processes are the same and the invention works for multiple species of mammals in the same way as it does in cattle.

INTERSPECIES EFFECTS

The invention being universal for mammals in superovulation success, each species of FSH would work in another species. The following data using equine, ovine and porcine FSH in cows is shown in the following table, Table 19. It shows that FSH of three species (equine, ovine and porcine) is effective in producing superovulation in a fourth species (bovine).

TABLE 19

Species	# Cows	# Transferable embryo	# Total embryos
Equine	158	4.67	5.76 ± 6.29
Ovine	82	4.74	5.96 ± 5.84
Porcine	972	5.27	9.48 ± 8.49

Sheep are seasonable breeders which is a disadvantage in fat lamb production because it produces seasonal supplies. It is advantageous to produce lambs at other times of the year. These out of season lambs can only be produced if the ewes can be induced to breed out of season. The physiological processes involved in induction of estrus out of season are the same as those used in superovulation. Thus an experiment was designed to compare those of FSH with a low LH content (FSH/LH > 1000, SUPER-OV, the trade-mark of a composition containing FSH with a low LH content) with regular FSH-P (FSH/LH < 500). The experiment was performed in New Zealand by officers of Waitaki International and supervised by officers of the Ministry of Agriculture and Fisheries. The aim in and out of season breeding is to produce two ovulations per ewe. The is a distinction from superovulation when ovulation rate is maximized and the embryos are harvested. In out of season breeding the ewes are mated with rams and the embryos develop in the ewe without harvesting allowing for normal gestation of the embryo.

Thirty-eight ewes aged about five years were treated at random with either 2.5 units FSH (SUPER-OV, FSH/LH > 1000) or 2.5 units FSH-P (Burns Biotec Omaha Nebraska)(FSH/H < 500). The ewes were pretreated with vaginal sponges containing a progestogen (The Upjohn Co., Kalamazoo, MI). The sponges were withdrawn four days after FSH treatment and the ewes slaughtered four days later. The total ovulations on both ovaries were counted.

FSH produced 1.525 ± 0.6 ovulations, significantly more than the 0.154 ± 0.3 ovulations produced by FSH-P ($P<0.001$ by the TTest). A listing of the data and the TTest is presented in Table 20.

As FSH preparation with low levels of LH

An FSH preparation with low levels of LH (SUPER-OV FSH/LH > 1000) is a superior drug for out of season breeding of sheep to regular FSH-P with a high level of LH (FSH/LH < 500).

COMPARISON SUPER-OV WITH FSH-P
MARCH, 1987, DOSE 2.5 UNITS
Variable Under Analysis - # OVULATIONS
Variable used to Group Cases - TREATMENT

TABLE 20

FSH-P	
Number of cases	= 19
Mean	= 0.1053
Variance	= 0.0994
Standard deviation	= 0.3153
Standard error of the mean	= 0.0723
SUPER-OV	
Number of cases	= 19
Mean	= 1.5263
Variance	= 0.3743
Standard deviation	= 0.6118
Standard error of the mean	= 0.1404
T-Test statistics	
Difference (Mean X - Mean Y)	=-1.4211
Standard error of the difference	= 0.1579
t - statistic	= 9.0000
Degrees of freedom	= 36
Probability of t (One tailed test)	= 0.0000
Probability of t (Two tailed test)	= 0.0000

SUPEROVULATION IN GOATS

In November, 1987, 24 goats were superovulated with either 30 units SUPER-OV or 30 units FSH-P (Schering Corporation, Kenilworth NJ). The SUPER-OV had an FSH/LH ration > 1000 and the FSH-P < 500. The goats were pretreated with progesterone and treated for four days with one of the FSH preparations. They were bred when they came in estrus and the embryos recovered non-surgically six days after breeding. The recovered embryos were counted and classified as transferable (viable) or nontransferable.

SUPER-OV produced significantly more transferable embryos than the FSH-P. (8.7 ± 1.9 vs. 5.7 ± 3.6, P = 0.227). There was no difference in the total embryos collected (9.2 ± 2.1 vs. 7.8 ± 3.9). The response from FSH-P was more viable than from SUPER-OV.

SUPEROVULATION IN GOATS, WEST TEXAS
COMPARISON FSH-P AND SUPER-OV

TABLE 22

Subject	TREATMENT	DATE	DOSE (UNITS)	TRANSFERABLE EMBRYOS	TOTAL EMBRYOS
1	FSH-P	NOV87	30	6	14
2	FSH-P	NOV87	30	2	3
3	FSH-P	NOV87	30	6	6
4	FSH-P	NOV87	30	7	8
5	FSH-P	NOV87	30	1	3
6	FSH-P	NOV87	30	5	6
7	FSH-P	NOV87	30	8	10
8	FSH-P	NOV87	30	12	12
9	FSH-P	NOV87	30	0	2
10	FSH-P	NOV87	30	6	7
11	FSH-P	NOV87	30	5	10
12	FSH-P	NOV87	30	11	12
13	SUPER	NOV87	30	9	9
14	SUPER	NOV87	30	8	10
15	SUPER	NOV87	30	9	9
16	SUPER	NOV87	30	9	9
17	SUPER	NOV87	30	5	6
18	SUPER	NOV87	30	7	7
19	SUPER	NOV87	30	7	8

TABLE 22 Con't.

1340132

	Subject	TREATMENT	DATE	DOSE (UNITS)	TRANSFERABLE EMBRYOS	TOTAL EMBRYOS
5	20	SUPER	NOV87	30	9	9
	21	SUPER	NOV87	30	9	10
	22	SUPER	NOV87	30	12	12
	23	SUPER	NOV87	30	12	14
	24	SUPER	NOV87	30	8	8

10 SUPEROVULATION IN GOATS, WEST TEXAS

COMPARISON FSH-P AND SUPER-OV

Variable under analysis - TOTAL EMBRYOS

Variable used to group cases - TREATMENT

TABLE 23

15	Group 1 FSH-P	
	Number of cases	= 12
	Mean	= 7.7500
	Variance	= 15.4773
	Standard deviation	= 3.9341
20	Standard error of the mean	-1.1357
	Group 2 SUPER	
	Number of cases	= 12
	Mean	= 9.2500
	Variance	= 4.5682
25	Standard deviation	= 2.1373
	Standard error of the mean	= 0.6170
	T-Test statistics	
	Difference (Mean X - Mean Y)	=-1.5000
	Standard error of the difference	= 1.2925
30	t - statistic	= 1.1606
	Degrees of freedom	= 22
	Probability of t (One tailed test)	= 0.1291
	Probability of t (Two tailed test)	= 0.2583

SUPEROVULATION IN GOATS, WEST TEXAS
COMPARISON FSH-P AND SUPER-OV
Variable under analysis - TRANSFERABLE EMBRYOS
Variable used to group cases - TREATMENT

TABLE 24

Group 1 FSH-P	
Number of cases	= 12
Mean	= 5.7500
Variance	= 13.1136
Standard deviation	= 3.6213
Standard error of the mean	= 1.0454

Group 2 SUPER	
Number of cases	= 12
Mean	= 8.6667
Variance	= 3.8788
Standard deviation	= 1.9695
Standard error of the mean	= 0.5685

T-Test statistics	
Difference (Mean X - Mean Y)	=-2.9167
Standard error of the difference	= 1.1900
t - statistic	= 2.4510
Degrees of freedom	= 22
Probability of t (One tailed test)	= 0.0113
Probability of t (Two tailed test)	= 0.0227

Food and Drug Administration regulations require that any multidose injectable use a preservative for the prevention of bacterial contamination of the multidose vial. On page 1491 of the U.S.P. XXI (Pharmaceutic Ingredients/Reference Tables) as listed by categories under the heading of Antimicrobial Preservative is listed Thymol along with other preservative materials. Thymol was found to be one in the list of the preservatives that does not damage

glycoprotein hormones. In order to prove that Thymol can be used with the superovulation compound of the present invention as a preservative, it was established that 0.1% weight of Thymol does not harm FSH. A phosphate buffered saline was used as the diluent but other solutions such as normal saline would be safe to use. U.S.P. XXI, NF XVI Antimicrobial Preservative effectiveness tests were completed utilizing two levels of Thymol (0.08% and 0.04% by weight) against superovulation composition with diluent. Thymol at the 0.08% by weight was effective against all the test organisms and the 0.04% by weight was effective against four out of five.

Preservatives are substances added to dosage forms to protect them from microbial contamination. They are required to be added to multidose vials. Of the 24 antimicrobial preservatives listed on Page 1491 of U.S.P. XX 1, and U.S.P. and NF Pharmaceutical Ingredients, Thymol (5-methyl-2(1-methylethyl)phenol) was found to be compatible with FSH. Thymol is only slightly soluble in water, e.g., at 19°C 1.3g/litre and at 100°C 1.6 gm/litre. Thus a useful working solution at room temperature is a 1% solution. It was therefore decided to test the compatibility of 1% Thymol in phosphate buffered saline on the activity of FSH over a period of four days, the recommended life of a multidose vial of SUPER-OV solution. SUPER-OV is a follicle stimulating hormone preparation that is used in inducing superovulation in mammals prior to estrus induction and subsequent insemination, embryo collection and transfer.

The purpose of this study was to evaluate the effects of 1% Thymol on the integrity of the biological activity of FSH when used in SUPER-OV DILUENT is used to dissolve SUPER-OV for use in divided doses over a four day period. The objective was to define the FSH activity of SUPER-OV after being in a solution of PBS or PBS and

1% Thymol for four days. PBS was made up to make a solution containing Sodium chloride 8%, Potassium chloride 0.02%, Potassium phosphate 0.02% and Sodium phosphate 0.102%. One vial each containing 75 units of FSH were used.

The experiment was started on Wednesday $=t_0$

ended on the following Sunday $=t_4$

One vial of FSH containing 75 units was dissolved in 6 ml PBS (12 units/ml). Two samples of 2.5 ml were taken and to each another 2.5 ml of PBS was added. To one subsample was added 60 lambda Thymol in Ethanol.

The ninth dilution of the A_0 0 day, A_t 4 day, A_t 0 day and A_t 4 day samples were assayed by the radioligand assay (Bousfield & Ward, 1984).

STATISTICS:

The $i.d._{50}$ plus or minus the standard deviation in uUnits/tube will be calculated for each parameter for the logistic $F(x) = D + \frac{A - D}{1+(X/C)^B}$

where

A = cpm at zero dose (counts per minute), i.e. the binding response in the absence of added unlabeled hormone.

B = slope factor of the response curve.

C = $i.d._{50}$. The dose which displaces 50% of the specifically bound cpm ($i.d._{50}$).

D = cpm at infinite dose, i.e. the binding in the presence of an infinite does of unlabeled ligand.

X = dose of unlabelled ligand.

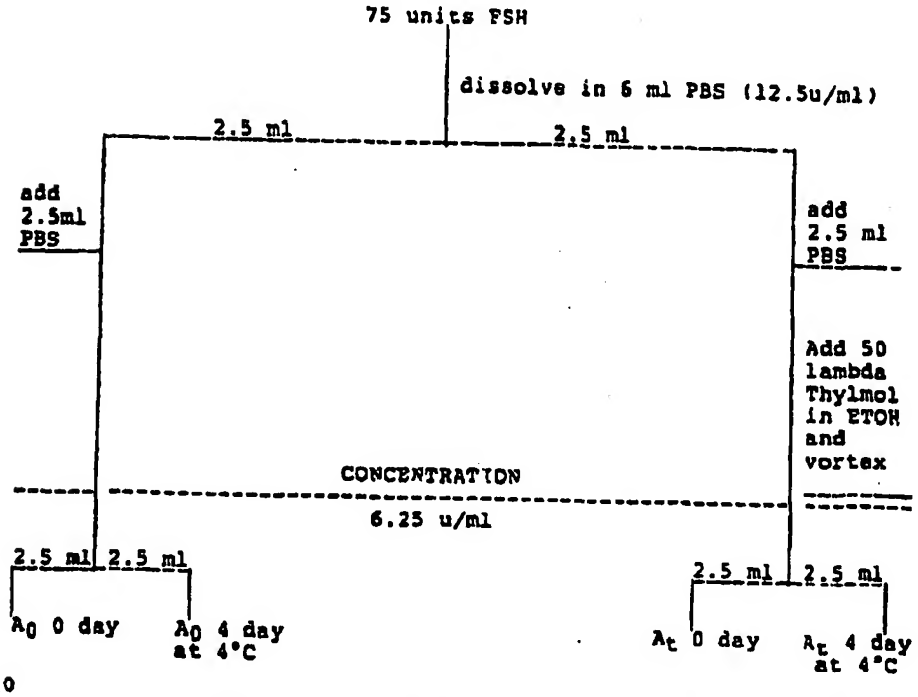
F(x) = the binding response at a given dose of X (cpm).

This equation is utilized along with a program Curve fit routine (Newton Gauss best for analysis approach) for the analysis of the radioreceptor dose response curve. The Percent Total Specific Binding was plotted against Log Dose (mU/tube) for each FSH sample

with and without Thymol preservative comparing the FSH standard used in the assay, the 0 and the 4 day result. The null hypothesis is that each of these curves will be the same.

Each 5 ml sample was further subdivided into two 2.5 ml samples, one to serve as the T_0 sample and the other as the t_4 sample. The following flow diagram represents this procedure.

FLOW DIAGRAM



The 0 day samples were frozen immediately, and the 4 day samples after 4 days at 4°C.

At the time of assay the samples were thawed, diluted 1/10 with RLA buffer and the following serial

dilutions made:

Serial Dilution #	Concentration	x50 lambda = Dose
1	0.635U/ML	0.031
2	0.31	0.016
3	0.16	0.008
4	0.08	0.004
5	0.04	0.002
6	0.02	0.001
7	0.01	0.0005
8	0.005	0.00025
9	0.0025	0.00013

The treatments effects were compared, and the null hypothesis tested using the T-Test for matched pairs.

The FSH activity (units of NIH-FSH-S1/mg) for 4 treatments in 3 lots of SUPER-OV are listed in Table 25.

TABLE 25

FSH ACTIVITY AFTER DISSOLVING PBS AND PBS + 1% THYMOL

Lot #	PBS		THYMOL	
	Day 0	Day 4	Day 0	Day 4
603	0.85	0.61	0.53	0.67
700	0.94	1.37	1.30	0.84
706	0.58	0.65	0.53	0.53

TTests for matched pairs were conducted to compare Thymol day 0 vs. Thymol Day 4 (P=0.615 for null hypothesis Table 26), PBS day 0 vs. PBS day 4 (P=0.694, Table 27), 0 days vs. 4 days (P=0.938, Table 28) and PBS vs. Thymol (P=0.534, Table 29).

The percent total specific binding has been plotted against log dose for each FSH batch in PBS with and without Thymol comparing 0 days, 4 days and the NIH-FSH-S8 standard (Figures 2 to 6). The curves are nearly identical in each case indicting that there has been no change in FSH activity by putting it in PBS solution with

PBS or PBS containing 1% Thymol does not effect the FSH activity of SUPER-OV over a four day period making 1% Thymol a potential preservative for use in SUPER-OV DILUENT.

TABLE 26

TTEST COMPARING FSH POTENCY IN THYMOL AT 0 AND 4 DAYS

THYMOL 0 TIME	
Mean	= 0.7867
Variance	= 0.1976
Standard deviation	= 0.4446
Standard error of the mean	= 0.3144
THYMOL 4 DAYS	
Mean	= 0.6800
Variance	= 0.0241
Standard deviation	= 0.1552
Standard error of the mean	= 0.1098
T-Test statistics	
Difference (Mean X- Mean Y)	= 0.1070
Standard error of the difference	= 0.1812
t - statistic	= 0.5886
Degrees of freedom	= 2
Probability of t (One tailed test)	= 0.3074
Probability of t (Two tailed test)	= 0.6148

TABLE 27

TTEST COMPARING FSH POTENCY IN PBS AT 0 AND 4 DAYS

PBS 0 TIME	
Mean	= 0.7900
Variance	= 0.0351
Standard Deviation	= 0.1873
Standard error of the mean	= 0.1325

PBS 4 DAYS

Mean	= 0.8767
Variance	= 0.1829
Standard deviation	= 0.4277
Standard error of the mean	= 0.3024

T-Test statistics

Difference (Mean X - Mean Y)	= 0.0870
Standard error of the difference	= 0.1936
t - statistic	= 0.4477
Degrees of freedom	= 2
Probability of t (One tailed test)	= 0.3472
Probability of t (Two tailed test)	= 0.6943

TABLE 28

TTEST COMPARING FSH POTENCY AT 0 AND 4 DAYS

ZERODAYS

Mean	= 0.7883
Variance	= 0.0931
Standard deviation	= 0.3051
Standard error of the mean	= 0.1365

FOURDAYS

Mean	= 0.7783
Variance	= 0.0944
Standard deviation	= 0.3073
Standard error of the mean	= 0.1374

T-Test statistics

Difference (Mean X- Mean Y)	= 0.0100
Standard error of the difference	= 0.1262
t - statistic	= 0.0792
Degrees of freedom	= 5
Probability of t (One tailed test)	= 0.4690
Probability of t (Two tailed test)	= 0.9361

TABLE 29

TTEST COMPARING FSH POTENCY
IN PBS AND THYMOL

	PBS	
5	Mean	= 0.8333
	Variance	= 0.0895
	Standard Deviation	= 0.2991
	Standard error of the mean	= 0.1338
	THYMOL	
10	Mean	= 0.7333
	Variance	= 0.0921
	Standard deviation	= 0.3035
	Standard error of the mean	= 0.1357
	T-Test statistics	
15	Difference (Mean X - Mean Y)	= 0.1000
	Standard error of the difference	= 0.1256
	t - statistic	= 0.7961
	Degrees of freedom	= 5
	Probability of t (One tailed test)	= 0.2668
20	Probability of t (Two tailed test)	= 0.5337

1340132

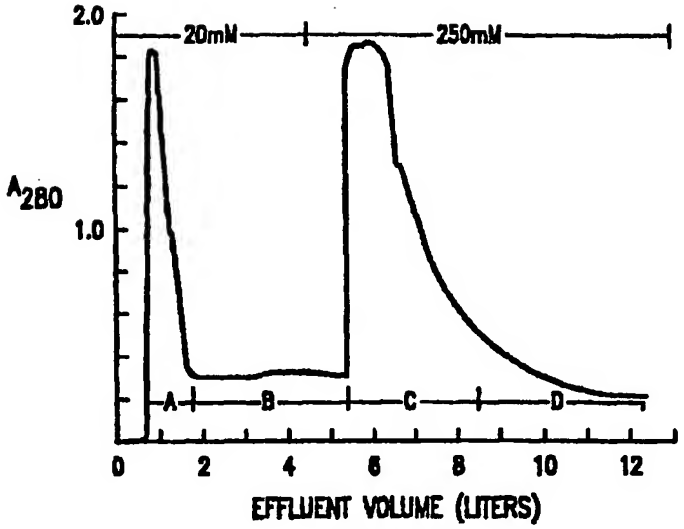


FIG. 1

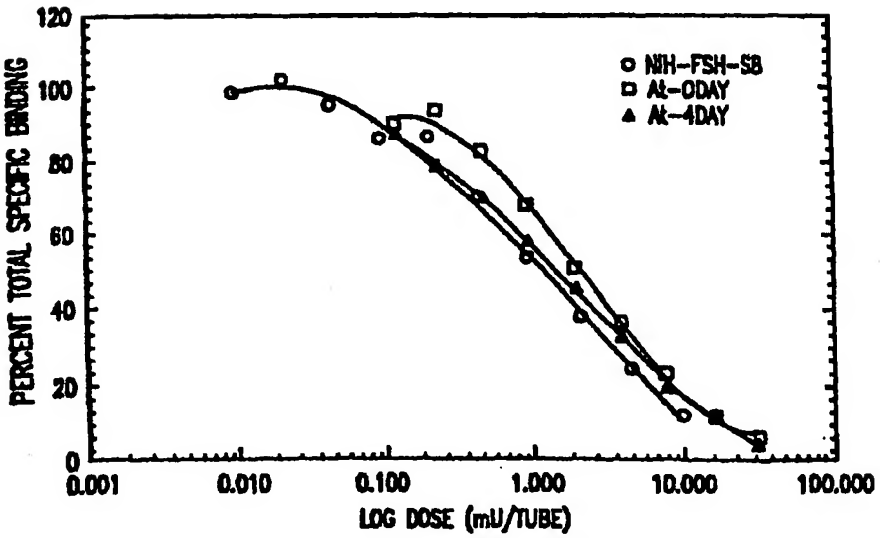


FIG. 2

Patent Agents
Smart & Biggar

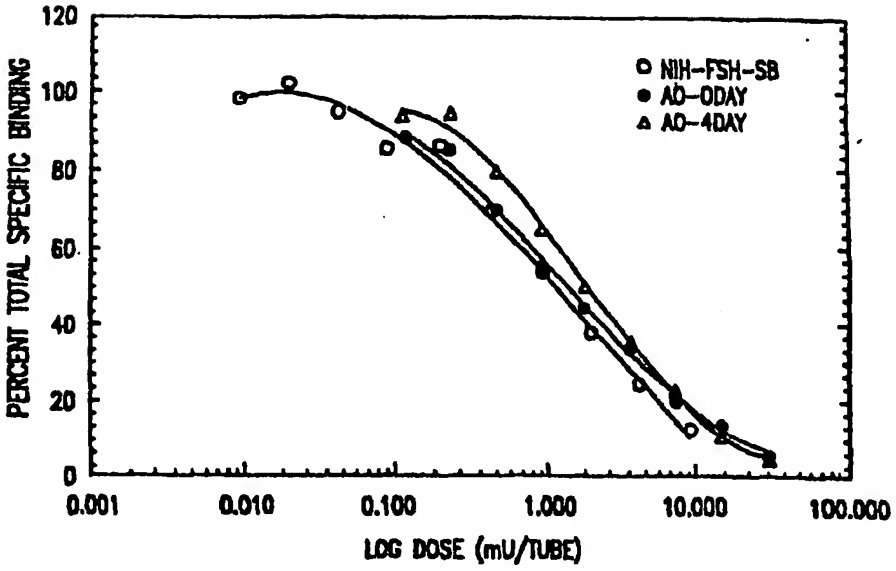


FIG. 3

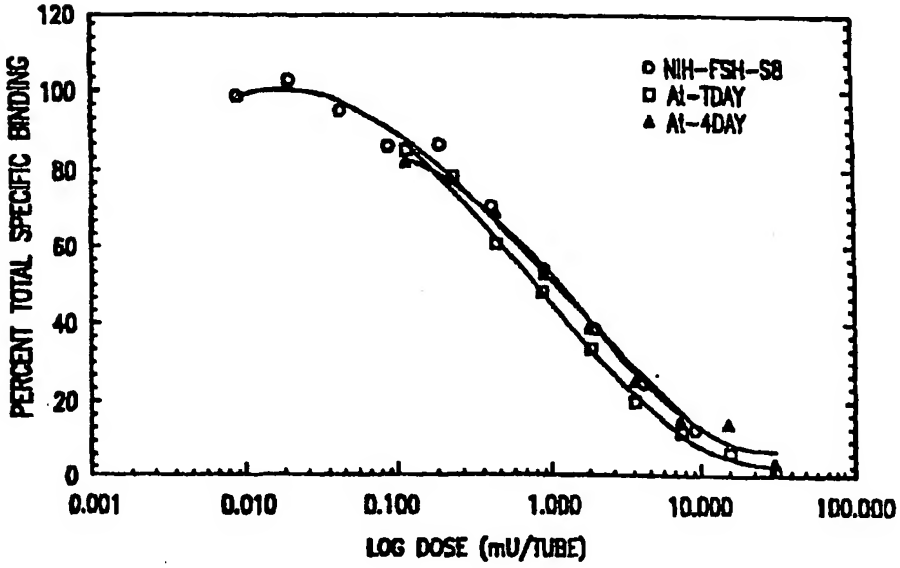


FIG. 4

Patent Agents
Smart & Biggar

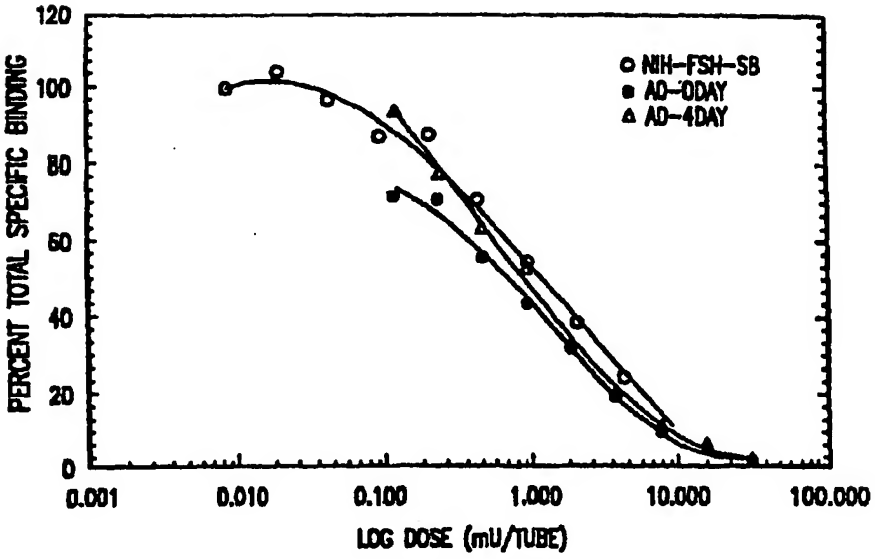


FIG. 5

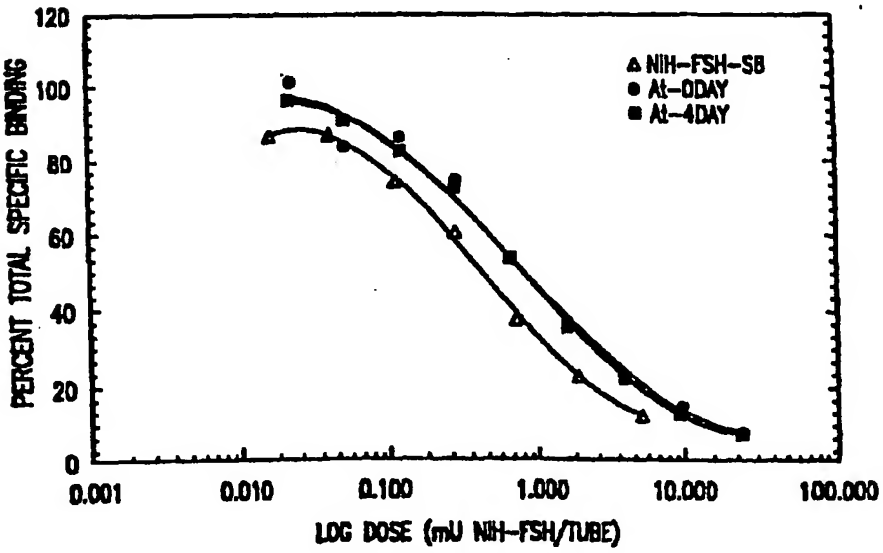


FIG. 6

Patent Agents
Smart & Biggar

1340132

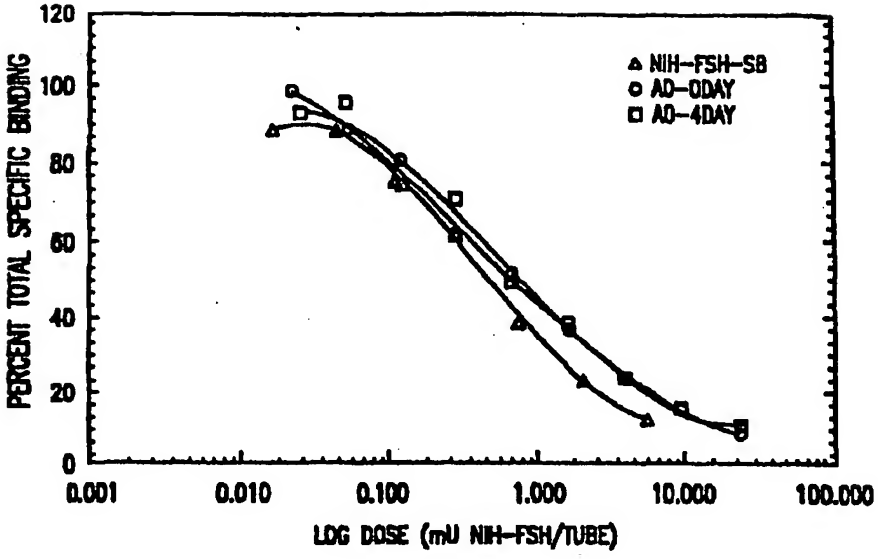


FIG. 7

CLAIMS: 54

1. A composition of matter for producing superovulation in cattle comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in cattle, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000.

2. A composition of matter according to claim 1, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 3000.

3. A composition of matter according to claim 1, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 2000.

4. A composition of matter according to claim 1, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

5. A composition of matter according to claim 1, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of from 2000 to 1655.

6. A composition of matter according to claim 1, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 1610 to 1.

7. A composition of matter according to claim 1, wherein said composition is a porcine pituitary hormone composition.

8. Use of a composition of matter comprising follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000, for the new use of producing superovulation in cattle.

9. Use as claimed in claim 8 by parenteral injection of said composition.

10. Use as claimed in claim 8 by parenteral injection of 75 units of said composition.

11. Use as claimed in claim 8 by parenteral injection of 75 units in 8 equal doses at approximately 12 hour intervals of said composition.

12. Use as claimed in claims 8, 9 or 10 of said composition, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 3000.

13. Use as claimed in claims 8, 9 or 10 of said composition, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 2000.

14. Use as claimed in claims 8, 9 or 10 of said composition, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

15. Use as claimed in claims 8, 9 or 10 of said composition, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1655.

16. Use as claimed in claims 8, 9 or 10 of said composition, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 1610 to 1.

17. Use as claimed in claim 8 of said composition, wherein said composition is a porcine pituitary hormone composition.

CLAIMS SUPPORTED BY THE SUPPLEMENTARY DISCLOSURE:

18. A composition of matter for producing superovulation in goats comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in goats, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000 to 1 or greater.

19. A composition of matter for producing superovulation in swine comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in swine, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000 to 1 or greater.

20. A composition of matter for producing superovulation in sheep comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in sheep, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000 to 1 or greater.

21. A composition of matter according to claims 18, 19 or 20, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 3000.

22. A composition of matter according to claims 18, 19 or 20, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 2000.

23. A composition of matter according to claims 18, 19 or 20, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

24. A composition of matter according to claims 18, 19 or 20, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1655.

25. A composition of matter according to claims 18, 19 or 20, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 1610 to 1.

26. A composition of matter for producing superovulation in mammals comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in mammals, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of 500 to 50,000 to 1, wherein, on a molecular weight basis, the luteinizing hormone is present at 16% by weight at the 500 to 1 ratio and .16% by weight at the 50,000 to 1 ratio.

27. The composition of matter according to claim 26, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 3000.

28. The composition of matter according to claim 26, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 2000.

29. The composition of matter according to claim 26, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

30. The composition of matter according to claim 26, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1655.

31. The composition of matter according to claim 26, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1610.

32. A composition of matter for producing superovulation in horses comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in horses, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000 to 1 or greater.

33. A composition of matter for producing superovulation in humans comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in humans, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of 500 to 30,000 to 1 or greater.

34. A composition of matter for producing superovulation in exotic mammals comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in exotic mammals, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range from 500 to 30,000 to 1 or greater.

35. A composition of matter according to claims 32, 33 or 34, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 3000.

36. A composition of matter according to claims 32, 33 or 34, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 2000.

37. A composition of matter according to claims 32, 33 or 34, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

38. A composition of matter according to claims 32, 33 or 34, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1655.

39. A composition of matter according to claims 32, 33 or 34, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1610.

40. A pharmaceutical composition for producing superovulation in mammals comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in mammals, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in

a range from 500 to 30,000 to 1 or greater; said pharmaceutical composition being in an aqueous solution with saline or phosphate buffered saline and an antimicrobial preservative which is compatible with the pharmaceutical composition.

41. The pharmaceutical composition according to claim 40, wherein said antimicrobial preservative comprises of 5-methyl-2(1-methylethyl) phenol.

42. The pharmaceutical composition according to claim 40, wherein said antimicrobial preservative, comprises at least 0.04% by weight or greater.

43. The pharmaceutical composition according to claim 40, wherein said antimicrobial preservative comprises from 0.5% by weight up to 0.1% by weight of the pharmaceutical composition solution.

44. The pharmaceutical composition according to claims 40, 41 or 42, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of from 500 to 30,000.

45. The pharmaceutical composition of claims 40, 41 or 42, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 3000.

46. The pharmaceutical composition of claims 40, 41 or 42, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 2000.

47. The pharmaceutical composition of claims 40, 41 or 42, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

48. The pharmaceutical composition of claims 40, 41 or 42, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1655.

49. The pharmaceutical composition of claims 40, 41 or 42, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 1610 to 1.

50. An injectable pharmaceutical composition for producing superovulation in mammals comprising:

an effective amount of follicle stimulating hormone and luteinizing hormone in said composition for producing superovulation in mammals, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000 to 1 or greater; said injectable pharmaceutical composition being in solution with saline, or phosphate-buffered saline, and an antimicrobial preservative which is compatible with the injectable pharmaceutical composition.

51. The injectable pharmaceutical composition according to claim 50, wherein said antimicrobial preservative comprises at least 0.04% by weight of the solution.

52. The injectable pharmaceutical composition according to claim 51, wherein said antimicrobial preservative comprises from 0.04 to 0.1% by weight of the solution.

53. The injectable pharmaceutical composition according to claim 51, wherein said antimicrobial preservative comprises 5-methyl-2(1-methylethyl)phenol.

54. The injectable pharmaceutical composition according to claims 50, 51 or 53, wherein the ratio of follicle stimulating hormone to luteinizing hormone is in a range up to 3000.

55. The injectable pharmaceutical composition according to claims 50, 51 or 53, wherein the ratio of follicle stimulating hormone to luteinizing hormone is in a range up to 2000.

56. The injectable pharmaceutical composition according to claims 50, 51 or 53, wherein the ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

57. The injectable pharmaceutical composition according to claims 50, 51 or 53, wherein the ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1655.

58. The injectable pharmaceutical composition according to claims 50, 51 or 53, wherein the ratio of follicle stimulating hormone to luteinizing hormone is 1610 to 1.

59. Use of a composition comprising follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is a range of from 500 to 30,000 to 1 or greater, for the new use of producing superovulation in mammals.

60. Use as claimed in claim 59 by parenteral injection of said composition.

61. Use as claimed in claim 59 by parenteral injection in 8 equal doses at approximately 12 hour intervals of said composition.

62. Use as claimed in claims 59, 60 or 61 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 3000.

63. Use as claimed in claims 59, 60 or 61 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 2000.

64. Use as claimed in claims 59, 60 or 61 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

65. Use as claimed in claims 59, 60 or 61 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1655.

66. Use as claimed in claims 59, 60 or 61 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 1610 to 1.

67. Use of a composition of matter comprising an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30;000 to 1 or greater, for the new use of enhancing twinning in sheep.

68. Use of a composition of matter comprising an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating

hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000 to 1 or greater, for the new use of enhancing twinning in cattle.

69. Use of a composition of matter comprising an effective amount of follicle stimulating hormone and luteinizing hormone, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is in a range of from 500 to 30,000 to 1 or greater, for the new use of achieving out of season breeding for mammals characterized as having breeding seasons.

70. Use as claimed in claims 67, 68 or 69 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 3000.

71. Use as claimed in claims 67, 68 or 69 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of up to 2000.

72. Use as claimed in claims 67, 68 or 69 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 2652 to 1.

73. Use as claimed in claims 67, 68 or 69 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is in a range of 1000 to 1655.

74. Use as claimed in claims 67, 68 or 69 of said composition of matter, wherein said ratio of follicle stimulating hormone to luteinizing hormone is 1610 to 1.

75. The composition of matter according to claim 18, wherein the ratio of follicle stimulating hormone in NIH-FSH-1 units to luteinizing hormone in NIH-LH-1 units is greater than 1,000.

76. The composition of matter according to claim 19, wherein the ratio of follicle stimulating hormone in NIH-FSH-1 units to luteinizing hormone in NIH-LH-1 units is greater than 1,000.

77. The composition of matter according to claim 20, wherein the ratio of follicle stimulating hormone in NIH-FSH-1 units to luteinizing hormone in NIH-LH-1 units is greater than 1,000.

78. The composition of matter according to claim 26, wherein the ratio of follicle stimulating hormone in NIH-FSH-1 units to luteinizing hormone in NIH-LH-1 units is greater than 1,000.

79. The composition of matter according to claim 26, wherein said composition is a porcine pituitary hormone composition.

80. The composition of matter according to claim 40, wherein the ratio of follicle stimulating hormone in NIH-FSH-1 units to luteinizing hormone in NIH-LH-1 units is greater than 1,000.

81. The composition of matter according to claim 40, wherein said composition is a porcine pituitary hormone composition.

82. Use as claimed in claim 59 of said composition of matter, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is greater than 1,000.

83. Use as claimed in claim 59 of said composition, wherein said composition is a porcine pituitary hormone composition.

84. Use as claimed in claim 67 of said composition of matter, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is greater than 1,000.

85. Use as claimed in claim 68 of said composition of matter, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is greater than 1,000.

86. Use as claimed in claim 69 of said composition of matter, wherein the ratio of follicle stimulating hormone in NIH-FSH-S1 units to luteinizing hormone in NIH-LH-S1 units is greater than 1,000.

The present invention relates to a composition and method for producing an optimum superovulation response in cattle.

In cattle, the fetal or neonatal female produces thousands of oocytes which are never fertilied. A multimillion dollar industry has developed that is concerned with methods to fertilie and transfer these oocytes to surrogate mothers. The advantages of such procedures include increasing the reproductive rate of valuable cows, decreasing the generation interval, progeny testing females, using superior females as donors, increasing the number of progeny per female through controlled multiple births, and transporting embryos with selected genetic characteristics to distant places.

The all important first step in these procedures is to produce a superovulation response in a superior female donor. The objective of superovulation is to increase the number of normal fertile eggs or embryos per donor. The basic principle of superovulation os to stimulate extensive follicular development through intramuscular or subcutaneous administration of a preparation having follicle-stilulating hormone (FSH) activity at levels in excess of normal endogenous levels. The most commonly used sources for this preparation are swine pituitary extracts or pregnant mares'